

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 March 2002 (07.03.2002)

PCT

(10) International Publication Number
WO 02/18545 A1

(51) International Patent Classification⁷: C12N 5/00,
5/02, 15/63, 15/85, 15/87

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(21) International Application Number: PCT/US01/27256

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(22) International Filing Date: 31 August 2001 (31.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/654,462 1 September 2000 (01.09.2000) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,
ZA, ZW.

(63) Related by continuation (CON) or continuation-in-part
(CIP) to earlier application:
US 09/654,462 (CIP)
Filed on 1 September 2000 (01.09.2000)

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

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Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

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*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

Applicants: Thomas M. Jessell et al.
Serial No.: 09/654,462
Filed: September 1, 2000
Exhibit 4

(54) Title: GENETIC DEMONSTRATION OF REQUIREMENT FOR NKX6.1, NKX2.2 AND NKX6.2 IN VENTRAL NEURON GENERATION

(57) Abstract: This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 or Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron. Provided are methods of diagnosing a motor neuron degenerative disease in a subject. Also provides is a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which is capable of expressing homeodomain Nkx6.1 or Nkx6.2 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

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**GENETIC DEMONSTRATION OF REQUIREMENT FOR
NKX6.1, NKX2.2 AND NKX6.2 IN VENTRAL NEURON GENERATION**

This application is a continuation-in-part of U.S. Serial No. 09/654,462, filed September 1, 2000, which is a continuation-in-part of U.S. Serial No. 09/569,259, filed May 11, 2000, the contents of which are hereby incorporated
5 by reference into the present application.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by
10 reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

15 BACKGROUND OF THE INVENTION

During the development of the embryonic central nervous system (CNS) the mechanisms that specify regional identity and neuronal fate are intimately linked (Anderson et al. 1997; Lumsden and Krumlauf 1996; Rubenstein et al. 1998).
20 In the ventral half of the CNS, for example, the secreted factor Sonic hedgehog (Shh) has a fundamental role in controlling both regional pattern and neuronal fate (Tanabe and Jessell 1996; Ericson et al. 1997; Hammerschmidt et al. 1997). Shh appears to function as a gradient signal.
25 In the spinal cord, five distinct classes of neurons can be generated in vitro in response to two- to threefold changes in the concentration of Shh, and the position at which each

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neuronal class is generated in vivo is predicted by the concentration required for their induction in vivo (Ericson et al. 1997a; Briscoe et al. 2000). Thus, neurons generated in more ventral regions of the neural tube
5 require progressively higher concentrations of Shh for their induction.

The genetic programs activated in neural progenitor cells in response to Shh signaling, however, remain incompletely
10 defined. Emerging evidence suggests that homeobox genes function as critical intermediaries in the neural response to Shh signals (Lumsden and Krumlauf 1996; Tanabe and Jessell 1996; Ericson et al. 1997; Hammerschmidt et al. 1997; Rubenstein et al. 1998). Several homeobox genes are
15 expressed by ventral progenitor cells, and their expression is regulated by Shh. Gain-of-function studies on homeobox gene action in the chick neural tube have provided evidence that homeodomain proteins are critical for the interpretation of graded Shh signaling and that they
20 function to delineate progenitor domains and control neuronal subtype identity (Briscoe et al. 2000). Consistent with these findings, the pattern of generation of neuronal subtypes in the basal telencephalon and in the ventral-most region of the spinal cord is perturbed in mice
25 carrying mutations in certain Shh-regulated homeobox genes (Ericson et al. 1997; Sussel et al. 1999; Pierani et al., unpublished).

Members of the *Nkx* class of homeobox genes are expressed by
30 progenitor cells along the entire rostro-caudal axis of the ventral neural tube, and their expression is dependent on

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Shh signaling (Rubenstein and Beachy 1998). Mutation in the *Nkx2.1* or *Nkx2.2* genes leads to defects in ventral neural patterning (Briscoe et al. 1999; Sussel et al. 1999), raising the possibility that *Nkx* genes play a key role in the control of ventral patterning in the ventral region of the CNS. Genetic studies to assess the role of *Nkx* genes have, however, focused on only the most ventral region of the neural tube. A recently identified *Nkx* gene, *Nkx6.1*, is expressed more widely by most progenitor cells within the ventral neural tube (Pabst et al. 1998; Qiu et al. 1998; Briscoe et al. 1999), suggesting that it may have a prominent role in ventral neural patterning. Here experiments show that in mouse embryos *Nkx6.1* is expressed by ventral progenitors that give rise to motor (MN), V2, and V3 neurons. Mice carrying a null mutation of *Nkx6.1* exhibit a ventral-to-dorsal switch in the identity of progenitor cells and a corresponding switch in the identity of the neuronal subtype that emerges from the ventral neural tube. The generation of MN and V2 neurons is markedly reduced, and there is a ventral expansion in the generation of a more dorsal V1 neuronal subtype. Together, these findings indicate that *Nkx6.1* has a critical role in the specification of MN and V2 neuron subtype identity and, more generally, that *Nkx* genes play a role in the interpretation of graded Shh signaling.

SUMMARY OF THE INVENTION

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

This invention also provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises:

- a) obtaining a nucleic acid sample from the subject; b) sequencing the nucleic acid sample; and c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

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This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises:

- a) obtaining a nucleic acid sample from the subject; b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes; c) separating the resulting nucleic acid fragments by size fractionation; d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of

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the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands; e) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates that the subject has the motor neuron degenerative disease.

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This invention provides a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which comprises an isolated nucleic acid molecule which is capable of expressing homeodomain Nkx6.1 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

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25 This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

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This invention provides a method of converting a stem cell

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into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.1 in the stem cell so as to thereby convert the stem cell into the ventral neuron.

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This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.2 in the stem cell so as to
10 thereby convert the stem cell into the ventral neuron.

This invention provides a method of diagnosing a neurodegenerative disease in a subject which comprises: a) obtaining a suitable sample from the subject; b)
15 extracting nucleic acid from the suitable sample; c) contacting the resulting nucleic acid with a nucleic acid probe, which nucleic acid probe (i) is capable of hybridizing with the nucleic acid of Nkx6.1 or Nkx6.2 and (ii) is labeled with a detectable marker; d) removing
20 unbound labeled nucleic acid probe; and e) detecting the presence of labeled nucleic acid, wherein the presence of labeled nucleic acid indicates that the subject is afflicted with a chronic neurodegenerative disease, thereby diagnosing a chronic neurodegenerative disease in the
25 subject.

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BRIEF DESCRIPTION OF THE FIGURESFigures 1A-1U

Selective changes in homeobox gene expression in ventral progenitor cells in *Nkx6.1* mutant embryos. (Figs. 1A-1C) Expression of *Nkx6.1* in transverse sections of the ventral neural tube of mouse embryos E9.5. (Fig. 1A) Expression of *Nkx6.1* is prominent in ventral progenitor cells and persists in some post-mitotic motor neurons at both caudal hindbrain, E10.5, (Fig. 1B) and spinal cord, E12.5, (Fig. 1C) levels. (Fig. 1D, and 1E) Summary diagrams showing domains of homeobox gene expression in wild-type mouse embryos (Fig. 1D) and the change in pattern of expression of these genes in *Nkx6.1* mutants (Fig. 1E), based on analyses at E10.0 - E12.5. (Figs. 1F-1I) Comparison of the domains of expression of *Nkx6.1* (Figs. 1F, 1J) *Dbx2* (Figs. 1G, 1H, 1K, 1L) and *Gsh1* (Figs. 1I, 1M) in the caudal neural tube of wild-type (Figs. 1F-1I) and *Nkx6.1* mutant (Figs. 1J-1H) embryos. (Fig. 1J) Horizontal lines, approximate position of dorsoventral boundary of the neural tube; vertical lines, expression of *Dbx2* and *Gsh1*. Expression of Sonic hedgehog, *Shh* (Figs. 1N, 1R), *Pax7* (Figs. 1N, 1R), *Nkx2.2* (Figs. 1O, 1S), *Pax6* (Figs. 1P, 1S), *Dbx1* (Figs. 1P, 1T) and *Nkx2.9* (Figs. 1Q, 1U) in wild-type (Figs. 1N-1Q) or *Nkx6.1* mutant (Figs. 1R-1U) embryos at spinal (Figs 1N-1P, 1R-1T) and caudal hindbrain levels (Figs 1Q, 1U). Arrowheads, approximate position of the dorsal limit of *Nkx6.1* expression. Scale bar shown in J= 100µm (Figs. 1A-1C); 50µm (Figs. 1F-1M) or 60µm (Figs. 1N-1U).

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Figure 2A-2T

Disruption of motor neuron differentiation in *Nkx6.1* mutant embryos. The relationship between the domain of *Nkx6.1* expression (Figs. 2A-2C, green) by ventral progenitors and the position of generation of motor neurons and V2 interneurons (Figs. 2A-2D) in the ventral spinal cord of E10.5 wild-type embryos. (Fig. 2A) *Isl1/2* motor neurons; (Fig. 2B) HB9 motor neurons; (Fig. 2C) *Lhx3* (*Lim3*) expression (red) by motor neurons, V2 interneurons and their progenitors is confined to the *Nkx6.1* progenitor domain. (Fig. 2D) *Chx10* (green) V2 interneurons coexpress *Lhx3* (red). Expression of *Isl1/2* (Figs. 2E, 2I), HB9 (Figs. 2F, 2J), *Lhx3* (Figs. 2G, 2K) and *Phox2a/b* (Figs. 2H, 2L) in the ventral spinal cord (Figs. 2E, 2F, 2G) and caudal hindbrain (Fig. 2H) of E10.5 wild-type (Figs. 2E-2H) and *Nkx6.1* mutant (Figs. 2I-2L) embryos. Pattern of expression of *Isl1/2* and *Lhx3* at cervical (Figs. 2M, 2N, 2Q, 2R) and thoracic (Figs. 2O, 2P, 2S, 2T) levels of E12.5 wild-type (Figs. 2M-2P) and *Nkx6.1* mutant (Figs. 2Q-2T) embryos. Arrows, position of *Isl1* dorsal D2 interneurons. (Figs. 10Q-10T) Absence, position of *Isl1/2* dorsal D2 interneurons. Scale bar shown in I = 60µm (Figs. 2A-2D); 80µm (Figs. 2E-2L); 120µm (Figs. 2M-2T).

Figures 3A-3J

Motor neuron subtype differentiation in *Nkx6.1* mutant mice. Depletion of both median motor column (MMC) and lateral motor column (LMC) neurons in *Nkx6.1* mutant mice. Expression of *Isl1/2* (red) and *Lhx3* (green) in E12.5 wild-type (Figs. 3A, 3C) and *Nkx6.1* mutant (Figs. 3B, 3D) mice spinal cord at forelimb levels (Figs. 3E-3J). Motor neuron

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generation at caudal hindbrain level (Figs. 3E, 3F) *Nkx6.1* expression in progenitor cells and visceral motor neurons in the caudal hindbrain (rhombomere [r] 7/8) of E10.5-E11 wild-type (Fig. 3E) *Nkx6.1* mutant (Fig. 3F) mice. *HB9* expression in hypoglossal motor neurons in E10.5-E11 wild-type mice (Fig. 3G) and *Nkx6.1* mutant (Fig. 3H) mice. Coexpression of *Isl1* (green) and *Phox2a/b* (red) in wild-type (Fig. 3I) or *Nkx6.1* mutant (Fig. 3J) mice. (h) hypoglossal motor neurons; (v) visceral vagal motor neurons. Scale bar shown in C = 50 μ m (Figs. 3A-3D) or 70 μ m (Figs. 3E-3J).

Figures 4A-4L

A switch in ventral interneuron fates in *Nkx6.1* mutant mice. *Chx10* expression in V2 neurons at rostral cervical levels of E10.5 wild-type (Fig. 4A) and *Nkx6.1* mutant (Fig. 4B) embryos. *En1* expression by V1 neurons at rostral cervical levels of wild-type (Fig. 4C) and *Nkx6.1* mutant (Fig. 4D) embryos. *Pax2* expression in a set of interneurons that includes V1 neurons ((Burrill et al. 1997) at caudal hindbrain levels of wild-type (Fig. 4E) and *Nkx6.1* mutant (Fig. 4F) embryos. (Figs. 4G and 4H) *Sim1* expression by V3 neurons in the cervical spinal cord of wild-type (Fig. 4G) and *Nkx6.1* mutant (Fig. 4H) embryos. *Evx1* expression by V0 neurons at caudal hindbrain levels of wild-type (Fig. 4I) and *Nkx6.1* mutant (Fig. 4J) embryos. *En1* (red) and *Lhx3* (green) expression by separate cell populations in the ventral spinal cord of E11 wild-type (Fig. 4K) and *Nkx6.1* mutant (Fig. 4L) embryos. Scale bar shown in B = 60 μ m (Figs. 4A-4D); 75 μ m (Figs. 4E, 4F); 70 μ m (Figs. 4G, 4J, 4H, 4J), 35 μ m (Figs. 4K and 4L).

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Figure 5A-5B

Changes in progenitor domain identity and neuronal fate in the spinal cord of *Nkx6.1* mutant embryos. (Fig. 5A). In wild-type mouse embryos, cells in the *Nkx6.1* progenitor domain give rise to three classes of ventral neurons: V2 neurons, motor neurons (MN) and V3 neurons. V3 neurons derive from cells in the ventral most region of *Nkx6.1* expression that also express *Nkx2.2* and *Nkx2.9*. V1 neurons derive from progenitor cells that express *Dbx2* but not *Nkx6.1*. (Fig. 5B). In *Nkx6.1* mutant embryos the domain of *Dbx2* expression by progenitor cells expands ventrally, and by embryonic day 12 [E12] occupies the entire dorsoventral extent of the ventral neural tube, excluding the floor plate. Checked area indicates the gradual onset of ventral *Dbx2* expression. This ventral shift in *Dbx2* expression is associated with a marked decrease in the generation of V2 neurons and motor neurons and a ventral expansion in the domain of generation of V1 neurons. A virtually complete loss of MN and V2 neurons is observed at cervical levels of the spinal cord. The generation of V3 neurons (and cranial visceral motor neurons at hindbrain levels) is unaffected by the loss of *Nkx6.1* or by the ectopic expression of *Dbx2*.

Figure 6

Human Homeobox Protein *Nkx6.1*. NCBI Accession No. P78426. (Inoue, H. et al., "Isolation, characterization, and chromosomal mapping of the human *Nkx6.1* gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Amino acid sequence of human homeobox protein *Nkx6.1*.

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Figure 7

Human NK Homeobox Protein (Nkx6.1) gene, exon 1. NCBI Accession No. U66797. Segment 1 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-682.

10 Figure 8

Human NK Homeobox Protein (Nkx6.1) gene, exon 2. NCBI Accession No. U66798. Segment 2 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-185.

Figure 9

20 Human NK Homeobox Protein (Nkx6.1) gene, exon 3 and complete cds. NCBI Accession No. U66799. Segment 3 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 25 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-273. Protein encoded is shown in Fig. 7.

Figure 10

30 Expression of Nkx6.2 and Nkx6.1 in developing mouse and chick spinal cord. (A) At e8.5, Nkx6.2 and Nkx6.1 are

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expressed in a broad ventral domain of the mouse neural tube. (B) At e9.0, *Nkx6.2* expression is largely confined to a narrow domain immediately dorsal to the domain of *Nkx6.1* expression. A few scattered cells that co-express *Nkx6.2* and *Nkx6.1* are detected in more ventral positions at this stage. (C) At e9.5, *Nkx6.2* is expressed in a narrow domain, dorsal to the *Nkx6.1* boundary. (D-G) Comparative patterns of expression of *Nkx6.2*, *Nkx6.1*, *Dbx2*, *Dbx1* and *Pax7* in the intermediate region of e10.5 mouse spinal cord. (H-L) Expression pattern of *Nkx6.2*, *Nkx6.1*, *Dbx2*, *Dbx1* and *Pax7* in HH stage 20 chick spinal cord. Panels on right indicate progenitor domains, defined according to Briscoe et al., 2000.

15 Figure 11

Elevation in *Nkx6.2* and *Dbx2* expression in p1 domain cells in *Nkx6.2* mouse mutants. (A) Diagram of the targeting construct (i) used to replace the coding sequence of *Nkx6.2* (ii) with a tau-lacZ PGK-neo cassette (iii). Red bar indicates region used as probe in genotyping. (B-D) Sagittal view of e10.5 spinal cord showing LacZ expression, detected by X-gal staining, in wild type (wt) (B) *Nkx6.2*^{+/tlz} (C) and *Nkx6.2*^{tlz/tlz} (D) embryos. (E-G) *Nkx6.2* and LacZ expression in the p1 domain of wt (E), *Nkx6.2*^{+/tlz} (F), and *Nkx6.2*^{tlz/tlz} (G) embryos at e10.5. (H-J) In situ hybridization with a 5'-UTR probe shows that expression of *Nkx6.2* is elevated in the p1 domain of *Nkx6.2*^{tlz/tlz} embryos (J), compared with wt (H) or *Nkx6.2*^{+/tlz} (I) embryos. (K-M) Expression of *Dbx2* is up regulated ~2- fold in cells within the p1 domain (yellow bracket) in *Nkx6.2*^{tlz/tlz} embryos (M), compared with wt (K), or *Nkx6.2*^{+/tlz} (L) embryos. Abbreviations in (A): H=

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HindIII, B= BamHI, N= NcoI, S= SphI, A=AccI.

Figure 12

A partial switch from V1 to V0 neuronal fate in *Nkx6.2* mutant mice. (A-E) Expression of *Nkx6.2* (A), *Nkx6.1* (C, D), *Dbx1* (B, C, E), and *Pax7* (B) appears normal at caudal hindbrain levels of e10.5 *Nkx6.2^{+/tlz}* embryos. The expression of *Nkx6.1* (D) and *Dbx1* (E) abuts the ventral and dorsal boundaries of *LacZ* expression. (F-J) In e10.5 *Nkx6.2^{tlz/tlz}* embryos, expression of *Nkx6.1* (H, I) and *Pax7* (G) is unchanged but expression of *Dbx1* (F, G, H) is expanded ventrally into the p1 domain. Many ventral ectopic *Dbx1⁺* cells in *Nkx6.2^{tlz/tlz}* embryos express *LacZ* (J). (K-M) *Evx1/2⁺* V0 neurons are generated dorsal to *En1⁺* V1 neurons (K) and *LacZ⁺* cells (M) in *Nkx6.2^{+/tlz}* embryos. *En1⁺* neurons express *LacZ* in *Nkx6.2^{+/tlz}* (L) and *Nkx6.2^{tlz/tlz}* (O) embryos. (N-P) *Evx1/2⁺* V0 neurons are generated in increased numbers and at ectopic ventral positions in the caudal hindbrain of *Nkx6.2^{tlz/tlz}* embryos. (N) The number of *En1⁺* V1 neurons is reduced and the remaining *En1⁺* neurons are intermingled with ectopic *Evx1/2⁺* cells. (P) Many *Evx1/2⁺* neurons in *Nkx6.2^{tlz/tlz}* embryos co-express *LacZ*. (Q) Quantitation of *Evx1/2⁺* V0, and *En1⁺* V1, neurons at the caudal hindbrain of *Nkx6.2^{+/tlz}* and *Nkx6.2^{tlz/tlz}* embryos at e10.5. Counts from 12 sections, mean + S.D. In panels (A-P), the white arrowhead indicates the p0/p1 boundary.

Figure 13

Deregulated expression of *Nkx6.2* in *Nkx6.1* mutant mice, and similar patterning activities of *Nkx6* proteins in chick neural tube. (A) In e10.5 wt embryos, *Nkx6.2* expression is

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confined to the p1 progenitor domain. (B) In *Nkx6.1*^{-/-} embryos, scattered *Nkx6.2*⁺ cells are detected in the p2, pMN and p3 domains. (C) In *Nkx6.1*^{-/-} embryos, *Nkx6.2* is expressed in most progenitors in the p2, pMN and p3 domains. (D-F) Misexpression of *Nkx6.2* at high levels represses the expression of *Dbx1* (D) and *Dbx2* (E), but not *Pax7* (F). (G-P) Expression of *Nkx6.2* in dorsal positions of the chick neural tube result in ectopic dorsal generation of motor neurons, as indicated by ectopic induction of *Lim3* and *HB9* expression (G-I, L-N). Forced expression of *Nkx6.2* at high levels in the p0 and p1 progenitor domains promotes the ectopic generation of *Chx10*⁺ V2 neurons (J, K, O, P) and suppresses *Evx1/2*⁺ V0 (K, P) and *En1*⁺ V1 (J, O) neurons.

15 Figure 14

The deregulated expression of *Nkx6.2* underlies motor neuron generation in *Nkx6.1* mutants. (A) In e10.5 wt embryos, *Nkx6.2* expression is confined to the p1 domain and *Nkx6.1* is expressed in the p2, pMN and p3 domains. (B) No change in the expression of *Nkx6.1* is detected in *Nkx6.2*^{tlz/tlz} embryos. (C, D) In *Nkx6.1*^{-/-} and *Nkx6.1*^{-/-}; *Nkx6.2*^{+/-tlz} embryos, *Nkx6.2* expression is derepressed in the p2, pMN and p3 domains. (E) No expression of *Nkx6.2* or *Nkx6.1* protein is detected in *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tlz} embryos. (F, G) *HB9*⁺, *Isl1/2*⁺ motor neurons are generated in normal numbers in *Nkx6.2*^{tlz/tlz} embryos. The number of motor neurons is reduced by ~60% in *Nkx6.1*^{-/-} embryos (H), by ~80% in *Nkx6.1*^{-/-}; *Nkx6.2*^{+/-tlz} embryos (I) and by >90% in *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tlz} at cervical levels of e10.5 spinal cord (J). (K-M) At e12, the number of motor neurons of medial (MMC) (*Isl1*⁺, *Lim3*⁺) and lateral (LMC) (*Isl1*⁺) subtype identity is reduced in

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similar proportions in *Nkx6.1*^{-/-} and *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tlz} embryos. Lim3⁺ V2 neurons are missing in *Nkx6.1*^{-/-} embryos and *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tlz} embryos at this stage. (N-P) Quantitation of HB9⁺ and Isl1/2⁺ motor neurons at cervical and lumbar levels in wt, *Nkx6.2* and *Nkx6.1* single mutants and in *Nkx6.2*; *Nkx6.1* compound mutants at e10 and e12. Counts from 12 sections, mean + S.D.

Figure 15

10 Changes in class I protein expression and ventral interneuron generation in *Nkx6* mutants. (A-E) Expression of *Nkx6.1* and *Nkx6.2* in the spinal cord in different *Nkx6* mutant backgrounds at e10.5. (F-J) Spatial patterns of Pax7 and Dbx2 expression in different *Nkx6* mutant backgrounds.

15 Note that the level of Dbx2 expression in the pMN domain of *Nkx6.1*^{-/-}; *Nkx6.2*^{+/tlz} is very low, implying the existence of a pMN domain restricted gene that has the capacity to repress Dbx2 expression. Recent studies have provided evidence that the bHLH protein Olig2 possesses these properties (Novitsch et al., 2001).

20 (K-O) Spatial patterns of expression of Pax7 and Dbx1 in different *Nkx6* mutant backgrounds. (P-T) Spatial patterns of generation of Evx1/2⁺ V0 neurons and En1⁺ V1 neurons in different *Nkx6* mutant backgrounds. (Q) The generation of V0 neurons expands ventrally into the p1 domain in *Nkx6.2*^{tlz/tlz} mutants at caudal spinal levels. (R, A') The number of En1⁺ V1 neurons increases ~3-fold in the ventral spinal cord of *Nkx6.1*^{-/-} mutants, and ectopic Evx1/2⁺ cells are detected in position of the pMN domain in these mice (see also Sander et al., 2000). (S, T A') There is a progressive increase in Evx1/2⁺ V0 neurons and a loss of En1⁺ V1 neurons in the

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ventral spinal cord of *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} and *Nkx6.1*^{-/-};*Nkx6.2*^{tlz/tlz} embryos. (U,V,Z) The generation of *Evx1/2*⁺ V0 neurons correlates with the pattern of expression of *Dbx1* in progenitors in wt, *Nkx6.2*^{tlz/tlz} and *Nkx6.1*^{-/-};*Nkx6.2*^{tlz/tlz} mutant backgrounds. Note that only the most lateral progenitor cells express *Dbx1* in *Nkx6.1*^{-/-};*Nkx6.2*^{tlz/tlz} embryos, suggesting that expression of *Dbx1* in more medially-positioned progenitors is repressed by an as yet undefined gene. (X, Y) Ectopic ventral *Evx1*⁺ V0 neurons derive from *Dbx1*⁻ progenitors in *Nkx6.1*^{-/-} and *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} mutant embryos. *Chx10*⁺ V2 neurons are generated at normal numbers in *Nkx6.2*^{tlz/tlz} mutants, but are missing at spinal cord levels in *Nkx6.1*^{-/-}, *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} and *Nkx6.1*^{-/-};*Nkx6.2*^{tlz/tlz} mutants (A'; Figure 5, see Sander et al., 2000).

Figure 16

Dissociation of *Dbx* expression and V0 neuronal fate in mice with reduced *Nkx6* protein activity. (A) In e10.0 wt embryos, p0 progenitor cells express *Dbx1* and generate *Evx1/2*⁺ V0 neurons. (B) In e10.0 *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} embryos there is no change in the domain of expression of *Dbx1*, but *Evx1/2*⁺ V0 neurons are generated in lateral positions, along much of the ventral neural tube. (C, D) In *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} embryos examined at e10.0 many ectopic ventral *Evx1/2*⁺ neurons express *LacZ*. Framed area in (C) is shown at high magnification in (D) and indicates *Evx1/2*⁺ neurons that coexpress *LacZ*. (E) *Evx1/2*⁺ neurons located at the level of the pMN domain (bracket) derive from progenitors that express low or negligible levels of *Dbx2* mRNA. (F) Summary of *Dbx1* expression and V0 neuron

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generation in wt, *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} and *Nkx6.1*
;*Nkx6.2*^{tlz/tlz} embryos. The dissociation of *Dbx1* and *Evx1/2*
expression in *Nkx6.1*^{-/-};*Nkx6.2*^{+/^{tlz}} embryo suggests that
reduced *Nkx6* repressor activity is sufficient to repress
5 *Dbx1* but insufficient to repress *Evx1* expression.

Figure 17

Genetic interactions between *Nkx6* and *Dbx* proteins during
the assignment of motor neuron and interneuron fate in the
10 mouse neural tube. (A) Summary of domains of expression of
Nkx6.1 (6.1), *Nkx6.2* (6.2), *Dbx1* (D1) and *Dbx2* (D2) in the
ventral neural tube of wild type (wt) and different *Nkx6*
mutant embryos. (B) Regulatory interactions between *Nkx* and
15 *Dbx* proteins in the ventral neural tube. These
interactions result in different levels of *Nkx6* protein
activity in distinct ventral progenitor domains, and thus
promote the generation of distinct neuronal subtypes. For
details see text.

20 Figure 18

Human NK Homeobox Protein (*Nkx6.2*) gene, complete cds. NCBI
Accession No. AF184215.

25 Figure 19

Human Homeobox Protein *Nkx6.2*. NCBI Accession No. AAK13251.
Amino acid sequence of human homeobox protein *Nkx6.2*.

Figure 20

30 Comparison of Amino Acid Sequences of *Nkx6.2* Protein of
Various Species with Other *Nkx* Protein Sequences. mNk6.3 =

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mouse amino acid sequence of Nkx6.3 protein; rNkx6.1 = rat amino acid sequence of Nkx6.1 protein; mNkx6.2 = mouse amino acid sequence of Nkx6.2 protein; and cNkx6.2 = chick amino acid sequence of Nkx6.2 protein.

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DETAILED DESCRIPTION OF THE INVENTION

As used herein, the following standard abbreviations are used throughout the specification to indicate specific amino acids:

5

| | |
|---------------------|---------------------|
| A=ala=alanine | R=arg=arginine |
| N=asn=asparagine | D=asp=aspartic acid |
| C=cys=cysteine | Q=gln=glutamine |
| E=glu=glutamic acid | G=gly=glycine |
| 10 H=his=histidine | I=ile=isoleucine |
| L=leu=leucine | K=lys=lysine |
| M=met=methionine | F=phe=phenylalanine |
| P=pro=proline | S=ser=serine |
| T=thr=threonine | W=trp=tryptophan |

15

| | |
|----------------|--------------|
| Y=tyr=tyrosine | V=val=valine |
|----------------|--------------|

B=asx=asparagine or aspartic acid
Z=glx=glutamine or glutamic acid

20

As used herein, the following standard abbreviations are used throughout the specification to indicate specific nucleotides: C=cytosine; A=adenosine; T=thymidine; G=guanosine; and U=uracil.

25

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

30

In an embodiment of the above-described method of converting a stem cell into a ventral neuron, the nucleic

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acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell. In a further embodiment of the method, the nucleic acid is introduced by transfection or transduction. In another further
5 embodiment of the method, the ventral neuron is a motor neuron, a V2 neuron or a V3 neuron.

As used herein, the term "nucleic acid" refers to either DNA or RNA, including complementary DNA (cDNA), genomic DNA
10 and messenger RNA (mRNA). As used herein, "genomic" means both coding and non-coding regions of the isolated nucleic acid molecule. "Nucleic acid sequence" refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It
15 includes both replicating vectors, infectious polymers of DNA or RNA and nonfunctional DNA or RNA.

The nucleic acids of the subject invention also include nucleic acids coding for polypeptide analogs, fragments or
20 derivatives which differ from the naturally-occurring forms in terms of the identity of one or more amino acid residues (deletion analogs containing less than all of the specified residues; substitution analogs wherein one or more residues are replaced by one or more residues; and addition analogs,
25 wherein one or more residues are added to a terminal or medial portion of the polypeptide) which share some or all of the properties of the naturally-occurring forms.

The nucleic acid sequences include both the DNA strand
30 sequence that is transcribed into RNA, the complementary DNA strand, and the RNA sequence that is translated into

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protein. The nucleic acid includes both the full length nucleic acid sequence as well as non-full length sequences. It being further understood that the sequence includes the degenerate codons of the native sequence or sequences which
5 may be introduced to provide codon preference in a specific host cell.

As used herein, "protein", "peptide" and "polypeptide" are used to denote two or more amino acids linked by a peptidic
10 bond between the α -carboxyl group of one amino acid and the α -amino group of the next amino acid. Peptide includes not only the full-length protein, but also partial-length fragments. Peptides may be produced by solid-phase synthetic methods that are well-known to those skilled in
15 the art. In addition to the above set of twenty-two amino acids that are used for protein synthesis in vivo, peptides may contain additional amino acids, including but not limited to hydroxyproline, sarcosine, and γ -carboxyglutamate. The peptides may contain modifying groups
20 including but not limited to sulfate and phosphate moieties. Peptides can be comprised of L- or D-amino acids, which are mirror-image forms with differing optical properties. Peptides containing D-amino acids have the advantage of being less susceptible to proteolysis in vivo.

25 Peptides may be synthesized in monomeric linear form, cyclized form or as oligomers such as branched multiple antigen peptide (MAP) dendrimers (Tam et al. Biopolymers 51:311, 1999). Nonlinear peptides may have increased
30 binding affinity by virtue of their restricted conformations and/or oligomeric nature. Peptides may also

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be produced using recombinant methods as either isolated peptides or as a portion of a larger fusion protein that contains additional amino acid sequences.

5 Peptides may be chemically conjugated to proteins by a variety of well-known methods. Such peptide-protein conjugates can be formulated with a suitable adjuvant and administered parenterally for the purposes of generating polyclonal and monoclonal antibodies to the peptides of
10 interest. Alternatively, unconjugated peptides can be formulated with adjuvant and administered to laboratory animals for the purposes of generating antibodies. Methods for generating and isolating such antibodies are well-known to those skilled in the art.

15

The nucleic acids of the subject invention include but are not limited to DNA, RNA, mRNA, synthetic DNA, genomic DNA, and cDNA.

20 The nucleic acid sequence of the Nkx6.2 gene for various species may be found under the following NCBI Accession Nos.: human: AF184215; N55046; N50716N; H49739; H46204; H18874; mouse: BB449783; AV331479; BB358883; BB355466; L08074; and D.melanogaster: AF220236.

25

The amino acid sequence of the Nkx6.2 protein for various species may be found under the following NCBI Accession Nos.: AAK13251; MXKN2; MXKN1; S35304; T28492; AAF33780; P01524; P01523; 9GSSB; 17GSB; 1BH5D; 4GSSB; 1PGTB; 1GSUB;
30 1GNWB; 2GLRB; 1AGSB.

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As used herein, the term "introducing into a cell" includes but is not limited to transduction and transfection. Transfection can be achieved by calcium phosphate co-precipitates, conventional mechanical procedures such as
5 micro-injection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors or any other method known to one skilled in the art. This invention provides an antibody produced by the above method.

10 This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises:
a) obtaining a nucleic acid sample from the subject; b) sequencing the nucleic acid sample; and c) comparing the
15 nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron
20 degenerative disease.

In an embodiment of the above-described method of diagnosing a motor neuron degenerative disease in a subject the motor neuron degenerative disease is amyotrophic
25 lateral sclerosis or spinal muscular atrophy.

As used herein, the term "sample" includes but is not limited to tonsil tissue, lymph nodes, spleen, skin lesions, blood, serum, plasma, cerebrospinal fluid,
30 lymphocytes, urine, transudates, exudates, bone marrow cells, or supernatant from a cell culture.

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As used herein, "subject" means any animal or artificially modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. The subjects include but are not limited to mice, rats, dogs, guinea pigs, ferrets, rabbits, chicken and primates. In the preferred embodiment, the subject is a human being.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises:

- 10 a) obtaining a nucleic acid sample from the subject; b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes; c) separating the resulting nucleic acid fragments by size fractionation; d) hybridizing the resulting separated nucleic acid fragments
- 15 with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of the nucleic acid probe is labeled with a detectable marker,
- 20 and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands; e) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and f)
- 25 comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein
- 30 identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates

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that the subject has the motor neuron degenerative disease.

In an embodiment of the above-described method of diagnosing a motor neuron degenerative disease in a subject the nucleic acid is DNA. In a further embodiment of the above-described method the nucleic acid is RNA. In another embodiment the size fractionation in step (c) is effected by a polyacrylamide or agarose gel. In another embodiment the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label. In yet another embodiment the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

As used herein, "detectable marker" includes but is not limited to a radioactive label, or a calorimetric, a luminescent, or a fluorescent marker. As used herein, "labels" include radioactive isotopes, fluorescent groups and affinity moieties such as biotin that facilitate detection of the labeled peptide. Other labels and methods for attaching labels to compounds are well-known to those skilled in the art.

The phrase "specifically hybridizing" and the phrase "selectively hybridizing" describe a nucleic acid that hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. By selectively hybridizing it is meant that a nucleic acid binds to a given target in a manner that is detectable in a different manner from non-target sequence under high

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stringency conditions of hybridization. "Complementary", "antisense" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectively and specifically hybridize to a nucleic acid. Proper annealing conditions depend, for example, upon a nucleic acid's length, base composition, and the number of mismatches and their position on the nucleic acid, and must often be determined empirically. For discussions of nucleic acid design and annealing conditions for hybridization, see, for example, Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory, Vols. 1-3 or Ausubel, F., et al. (1987) *Current Protocols in Molecular Biology*, New York. The above hybridizing nucleic acids may vary in length. The hybridizing nucleic acid length includes but is not limited to a nucleic acid of at least 15 nucleotides in length, of at least 25 nucleotides in length, or at least 50 nucleotides in length.

This invention provides a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which comprises an isolated nucleic acid molecule which is capable of expressing homeodomain Nkx6.1 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain

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transcription factor Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

5 In one embodiment of the above method, the nucleic acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell. In another embodiment of the above method, the nucleic acid is introduced by transfection or transduction. In a further embodiment of the above method, the ventral neuron is a motor neuron.

10

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.1 in the stem cell so as to
15 thereby convert the stem cell into the ventral neuron. In one embodiment of the above method, the ventral neuron is a motor neuron, a V2 interneuron or a V3 interneuron.

This invention provides a method of converting a stem cell
20 into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.2 in the stem cell so as to thereby convert the stem cell into the ventral neuron. In one embodiment of the above method, the ventral neuron is
25 a motor neuron.

This invention provides a method of diagnosing a neurodegenerative disease in a subject which comprises: a) obtaining a suitable sample from the subject; b)
30 extracting nucleic acid from the suitable sample; c) contacting the resulting nucleic acid with a nucleic acid

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probe, which nucleic acid probe (i) is capable of hybridizing with the nucleic acid of Nkx6.1 or Nkx6.2 and (ii) is labeled with a detectable marker; d) removing unbound labeled nucleic acid probe; and e) detecting the presence of labeled nucleic acid, wherein the presence of labeled nucleic acid indicates that the subject is afflicted with a chronic neurodegenerative disease, thereby diagnosing a chronic neurodegenerative disease in the subject.

10

In one embodiment of the above method, the suitable sample is spinal fluid. In another embodiment of the above method, the nucleic acid is DNA. In a further embodiment of the above method, the nucleic acid is RNA.

15

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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FIRST SERIES OF EXPERIMENTSEXPERIMENTAL DETAILS5 A. Materials and MethodsGeneration of Nkx6.1 null mutation

A null mutation in *Nkx6.1* was generated by using gene targeting in 129-strain ES cells by excising an 800-bp NotI
10 fragment containing part of exon 1 and replacing it by a
PGK-neo cassette (Sander and German, unpubl.) Mutants were
born at Mendelian frequency and died soon after birth; they
exhibited movements only upon tactile stimulation.

15 Immunocytochemistry and in situ hybridization

Localization of mRNA was performed by in situ hybridization
following the method of Schaeren-Wiemers and Gerfin-Moser
(1993). The *Dbx2* riboprobe comprised the 5' EcoRI fragment
of the mouse cDNA (Pierani et al. 1999). Probes for other
20 cDNAs were cited in the text and used as described therein.
Protein expression was localized by indirect fluorescence
immunocytochemistry or peroxidase immunocytochemistry
(Briscoe et al. 1999; Ericson et al. 1997). *Nkx6.1* was
detected with a rabbit antiserum (Briscoe et al. 1999).
25 Antisera against *Shh*, *Pax7*, *Isl1/2*, *HB9*, *Lhx3*, *Chx10*,
Phox2a/b, *En1*, and *Pax2* have been described (Briscoe et al.
1999; Ericson et al. 1997). Fluorescence detection was
carried out using an MRC 1024 Confocal Microscope (BioRad).

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B. Results and Discussion

To define the role of *Nkx6.1* in neural development, we compared patterns of neurogenesis in the embryonic spinal cord and hindbrain of wild-type mice and mice lacking *Nkx6.1* (Sander et al. 1998). In wild-type embryos, neural expression of *Nkx6.1* is first detected at spinal cord and caudal hindbrain levels at about embryonic day 8.5 (E8.5; Qiu et al. 1998; data not shown), and by E9.5 the gene is expressed throughout the ventral third of the neural tube (Figure 1A). The expression of *Nkx6.1* persists until at least E12.5 (Figures 1B, 1C; data not shown). *Nkx6.1* expression was also detected in mesodermal cells flanking the ventral spinal cord (Figures 1B, 1C). To define more precisely the domain of expression of *Nkx6.1*, we compared its expressions with that of ten homeobox genes - *Pax3*, *Pax7*, *Gsh1*, *Gsh2*, *Irx3*, *Pax6*, *Dbx1*, *Dbx1*, *Dbx2* and *Nkx2.9* - that have been shown to define discrete progenitor cell domains along the dorsoventral axis of the ventral neural tube (Goulding et al. 1991; Valerius et al. 1995; Ericson et al. 1997; Pierani et al. 1999; Briscoe et al. 2000).

This analysis revealed that the dorsal boundary of *Nkx6.1* expression is positioned ventral to the boundaries of four genes expressed by dorsal progenitor cells: *Pax3*, *Pax7*, *Gsh1* and *Gsh2* (Figures 1I, 1N; and data not shown). Within the ventral neural tube, the dorsal boundary of *Nkx6.1* expression is positioned ventral to the domain of *Dbx1* expression and close to the ventral boundary of *Dbx2* expression (Figures 1G, 1H, and 1P). The domain of *Pax6* expression extends ventrally into the domain of *Nkx6.1*

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expression (Figure 10), whereas the expression of *Nkx2.2* and *Nkx2.9* overlaps with the ventral-most domain of *Nkx6.1* expression (Figures 10, 1Q).

5 To address the function of *Nkx6.1* in neural development, we analyzed progenitor cell identity and the pattern of neuronal differentiation in *Nkx6.1* null mutant mice (Sander et al. 1998). We detected a striking change in the profile of expression of three homeobox genes, *Dbx2*, *Gsh1* and *Gsh2*,
10 in *Nkx6.1* mutants. The domains of expression of *Dbx2*, *Gsh1* and *Gsh2* each expanded into the ventral neural tube (Figures 1K-1M; data not shown). At E10.5, *Dbx2* was expressed at high levels by progenitor cells adjacent to
the floor plate, but at this stage ectopic *Dbx2* expression
15 was detected only at low levels in regions of the neural tube that generate motor neurons (Figure 1K). By E12.5, however, the ectopic ventral expression of *Dbx2* had become more uniform, and now clearly included the region of motor neuron and V2 neuron generation (Figure 1L). Similarly, in
20 *Nkx6.1* mutants, both *Gsh1* and *Gsh2* were ectopically expressed in a ventral domain of the neural tube, and also in adjacent paraxial mesodermal cells (Figure 1M; data not shown).

25 The ventral limit of *Pax6* expression was unaltered in *Nkx6.1* mutants, although the most ventrally located cells within this progenitor domain expressed a higher level of *Pax6* protein than those in wild-type embryos (Figures 10, 1S). We detected no change in the patterns of expression
30 of *Pax3*, *Pax7*, *Dbx1*, *Irx3*, *Nkx2.2*, or *Nkx2.9* in *Nkx6.1* mutant embryos (Figures 1R-1U; data not shown).

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Importantly, the level of Shh expression by floor plate cells was unaltered in *Nkx6.1* mutants (Figures 1N and 1R). Thus, the loss of *Nkx6.1* function deregulates the patterns of expression of a selected subset of homeobox genes in ventral progenitor cells, without an obvious effect on Shh levels (Figures 1D, 1E). The role of Shh in excluding *Dbx2* from the most ventral region of the neural tube (Pierani et al. 1999) appears therefore to be mediated through the induction of *Nkx6.1* expression. Consistent with this view, ectopic expression of *Nkx6.1* represses *Dbx2* expression in chick neural tube (Briscoe et al. 2000). The detection of sites of ectopic *Gsh1/2* expression in the paraxial mesoderm as well as the ventral neural tube, both sites of *Nkx6.1* expression, suggests that *Nkx6.1* has a general role in restricting *Gsh1/2* expression. The signals that promote ventral *Gsh1/2* expression in *Nkx6.1* mutants remain unclear, but could involve factors other than Shh that are secreted by the notochord (Hebrok et al. 1998).

The domain of expression of *Nkx6.1* within the ventral neural tube of wild-type embryos encompasses the progenitors of three main neuronal classes: V2 interneurons, motor neurons and V3 interneurons (Goulding et al. 1991; Ericson et al. 1997; Qiu et al. 1998; Briscoe et al. 1999, 2000; Pierani et al. 1999; Figures 2A-2D). We examined whether the generation of any of these neuronal classes is impaired in *Nkx6.1* mutants, focusing first on the generation of motor neurons. In *Nkx6.1* mutant embryos there was a marked reduction in the number of spinal motor neurons, as assessed by expression of the homeodomain proteins *Lhx3*, *Isl1/2* and *HB9* (Arber et al. 1999; Tsuchida

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et al. 1994; Figures 2E-2L), and by expression of the gene encoding the transmitter synthetic enzyme choline acetyltransferase (data not shown). In addition, few if any axons were observed to emerge from the ventral spinal cord (data not shown). The incidence of motor neuron loss, however, varied along the rostrocaudal axis of the spinal cord. Few if any motor neurons were detected at caudal cervical and upper thoracic levels of *Nkx6.1* mutants analyzed at E11-E12.5 (Figures 2M, 2N, 2Q, 2R), whereas motor neuron number was reduced only by 50%-75% at more caudal levels (Figures 2O, 2P, 2S, 2T; data not shown). At all axial levels, the initial reduction in motor neuron number persisted at both E12.5 and p0 (Figures 2M-2T; data not shown), indicating that the loss of *Nkx6.1* activity does not simply delay motor neuron generation. Moreover, we detected no increase in the incidence of TUNEL⁺ cells in *Nkx6.1* mutants (data not shown), providing evidence that the depletion of motor neurons does not result solely from apoptotic death.

The persistence of some spinal motor neurons in *Nkx6.1* mutants raised the possibility that the generation of particular subclasses of motor neurons is selectively impaired. To address this issue, we monitored the expression of markers of distinct subtypes of motor neurons at both spinal and hindbrain levels of *Nkx6.1* mutant embryos. At spinal levels, the extent of the reduction in the generation of motor neurons that populate the median (MMC) and lateral (LMC) motor columns was similar in *Nkx6.1* mutants, as assessed by the number of motor neurons that coexpressed *Isl1/2* and *Lhx3* (defining MMC neurons, Figures

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3A, 3B) and by the expression of *Raldh2* (defining LMC neurons, Sockanathan and Jessell 1998; Arber et al. 1999; Figures 3C, 3D). In addition, the generation of autonomic visceral motor neurons was reduced to an extent similar to
5 that of somatic motor neurons at thoracic levels of the spinal cord of E12.5 embryos (data not shown). Thus, the loss of *Nkx6.1* activity depletes the major subclasses of spinal motor neurons to a similar extent.

10 At hindbrain levels, *Nkx6.1* is expressed by the progenitors of both somatic and visceral motor neurons (Figures 3E, 3F; data not shown). We therefore examined whether the loss of *Nkx6.1* might selectively affect subsets of cranial motor
neurons. We detected a virtually complete loss in the
15 generation of hypoglossal and abducens somatic motor neurons in *Nkx6.1* mutants, as assessed by the absence of dorsally generated HB9⁺ motor neurons (Figures 3G, 3H; data not shown, Arber et al. 1999; Briscoe et al. 1999). In contrast, there was no change in the initial generation of
20 any of the cranial visceral motor neuron populations, assessed by coexpression of *Isl1* and *Phox2a* (Briscoe et al. 1999; Pattyn et al. 1997) within ventrally generated motor neurons (Figures 3I, 3J; data not shown). Moreover, at rostral cervical levels, the generation of spinal accessory
25 motor neurons (Ericson et al. 1997) was also preserved in *Nkx6.1* mutants (data not shown). Thus, in the hindbrain the loss of *Nkx6.1* activity selectively eliminates the generation of somatic motor neurons, while leaving visceral motor neurons intact. Cranial visceral motor neurons,
30 unlike spinal visceral motor neurons, derive from progenitors that express the related *Nkx* genes *Nkx2.2* and

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Nkx2.9 (Briscoe et al. 1999). The preservation of cranial visceral motor neurons in *Nkx6.1* mutant embryos may therefore reflect the dominant activities of *Nkx2.2* and *Nkx2.9* within these progenitor cells.

5

We next examined whether the generation of ventral interneurons is affected by the loss of *Nkx6.1* activity. V2 and V3 interneurons are defined, respectively, by expression of *Chx10* and *Sim1* (Arber et al. 1999; Briscoe et al. 1999; Figures 4A, 4G). A severe loss of *Chx10* V2 neurons was detected in *Nkx6.1* mutants at spinal cord levels (Figure 4B), although at hindbrain levels of *Nkx6.1* mutants ~50% of V2 neurons persisted (data not shown). In contrast, there was no change in the generation of *Sim1* V3 interneurons at any axial level of *Nkx6.1* mutants (Figure 4H). Thus, the elimination of *Nkx6.1* activity affects the generation of only one of the two major classes of ventral interneurons that derive from the *Nkx6.1* progenitor cell domain.

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Evx1⁺, *Pax2*⁺ V1 interneurons derive from progenitor cells located dorsal to the *Nkx6.1* progenitor domain, (Figure 4B) within a domain that expresses *Dbx2*, but not *Dbx1* (Burrill et al. 1997; Matise and Joyner 1997; Pierani et al. 1999). Because *Dbx2* expression undergoes a marked ventral expansion in *Nkx6.1* mutants, we examined whether there might be a corresponding expansion in the domain of generation of V1 neurons. In *Nkx6.1* mutants, the region that normally gives rise to V2 neurons and motor neurons now also generated V1 neurons, as assessed by the ventral shift in expression of the *En1* and *Pax2* homeodomain

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proteins (Figures 4B, 4C, 4E, 4F). Consistent with this, there was a two- to threefold increase in the total number of V1 neurons generated in *Nkx6.1* mutants (Figures 4C, 4D). In contrast, the domain of generation of *Evx1/2* V0 neurons, which derive from the *Dbx1* progenitor domain (Pierani et al. 1999), was unchanged in *Nkx6.1* mutants (Figures 4I, 4J). Thus, the ventral expansion in *Dbx2* expression is accompanied by a selective switch in interneuronal fates, from V2 neurons to V1 neurons. In addition, we observed that some neurons within the ventral spinal cord of *Nkx6.1* mutants coexpressed the V1 marker *En1* and the V2 marker *Lhx3* (Figures 4K, 4L). The coexpression of these markers is rarely if ever observed in single neurons in wild type embryos (Ericson et al. 1996). Thus, within individual neurons in *Nkx6.1* mutants, the ectopic program of V1 neurogenesis appears to be initiated in parallel with a residual, albeit transient, program of V2 neuron generation. This result complements observations in *Hb9* mutant mice, in which the programs of V2 neuron and motor neuron generation coincide transiently within individual neurons (Arber et al. 1999; Thaler et al. 1999).

Taken together, the findings herein reveal an essential role for the *Nkx6.1* homeobox gene in the specification of regional pattern and neuronal fate in the ventral half of the mammalian CNS. Within the broad ventral domain within which *Nkx6.1* is expressed (Figure 5A), its activity is required to promote motor neuron and V2 interneuron generation and to restrict the generation of V1 interneurons (Figure 5B). It is likely that the loss of motor neurons and V2 neurons is a direct consequence of the

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loss of *Nkx6.1* activity, as the depletion of these two neuronal subtypes is evident at stages when only low levels of *Dbx2* are expressed ectopically in most regions of the ventral neural tube. Nonetheless, it can not be excluded that low levels of ectopic ventral *Dbx2* expression could contribute to the block in motor neuron generation. Consistent with this view, the ectopic expression of *Nkx6.1* is able to induce both motor neurons and V2 neurons in chick neural tube (Briscoe et al. 2000). V3 interneurons and cranial visceral motor neurons derive from a set of *Nkx6.1* progenitors that also express *Nkx2.2* and *Nkx2.9* (Briscoe et al. 1999, Figure 5A). The generation of these two neuronal subtypes is unaffected by the loss of *Nkx6.1* activity, suggesting that the actions of *Nkx2.2* and *Nkx2.9* dominate over that of *Nkx6.1* within these progenitors. The persistence of some spinal motor neurons and V2 neurons in *Nkx6.1* mutants could reflect the existence of a functional homologue within the caudal neural tube.

The role of *Nkx6.1* revealed in these studies, taken together with previous findings, suggests a model in which the spatially restricted expression of *Nkx* genes within the ventral neural tube (Figure 5) has a pivotal role in defining the identity of ventral cell types induced in response to graded *Shh* signaling. Strikingly, in *Drosophila*, the *Nkx* gene *NK2* has been shown to have an equivalent role in specifying neuronal fates in the ventral nerve cord (Chu et al. 1998; McDonald et al. 1998). Moreover, the ability of *Nkx6.1* to function as a repressor of the dorsally expressed *Gsh1/2* homeobox genes parallels the ability of *Drosophila* *NK2* to repress *Ind*, a *Gsh1/2*-like

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homeobox gene (Weiss et al. 1998). Thus, the evolutionary origin of regional pattern along the dorsoventral axis of the central nervous system may predate the divergence of invertebrate and vertebrate organisms.

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SECOND SERIES OF EXPERIMENTSIntroduction

5 During the development of the vertebrate central nervous system, the assignment of regional identity to neural progenitor cells has a critical role in directing the subtype identity of post-mitotic neurons. Within the ventral half of the neural tube, the specification of progenitor cell identity is initiated by the long-range signalling activity of the secreted factor, Sonic hedgehog (Shh) (Briscoe et al., 2001; Briscoe and Ericson, 2001). Shh signaling appears to establish ventral progenitor cell identities by regulating the spatial pattern of expression of homeodomain transcription factors of the Nkx, Pax, Dbx and Irx families (Ericson et al., 1997; Pierani et al., 1999; Briscoe et al., 2000). Members of all four gene families have been duplicated during evolution (Shoji et al., 1996; Wang et al., 2000; Hoshiyama et al., 1998, Peters et al., 2001), and the resulting homeodomain protein pairs are typically expressed in overlapping or nested domains within the neural tube (Briscoe and Ericson, 2001). Some of these homeodomain protein pairs have been proposed to have distinct, and others redundant, roles in spinal cord patterning (Mansouri and Gruss, 1998; Briscoe et al., 1999; Pierani et al., 2001), but the impact of such homeobox gene duplication on neuronal diversification has not been explored directly.

30 One unifying feature of this diverse array of progenitor homeodomain proteins is their subdivision into two general

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groups, termed class I and II proteins, on the basis of their mode of regulation by Shh signalling (Briscoe and Ericson, 2001). The class I proteins are constitutively expressed by neural progenitor cells, and their expression is repressed by Shh signaling, whereas neural expression of the class II proteins requires exposure to Shh (Ericson et al., 1997; Qiu et al., 1998; Briscoe et al., 1999; 2000; Pabst et al., 2000). Although the spatial pattern of expression of the class I proteins has revealed the existence of five ventral progenitor domains, class II proteins have been identified for only two of these domains (Briscoe et al., 2000), raising questions about the existence and identity of additional class II proteins.

There is, however, emerging evidence that the combination of class I and II proteins that is expressed by neural progenitor cells directs the fate of their neuronal progeny. In support of this, misexpression of individual progenitor homeodomain proteins in the chick neural tube promotes the ectopic generation of neuronal subtypes, with a specificity predicted by the normal profile of progenitor homeodomain protein expression (Briscoe et al., 2000; Pierani et al., 2001). Conversely, the analysis of mouse mutants has provided genetic evidence that the activities of specific class I and II proteins are required to establish progenitor cell domains and to direct ventral neuronal fates (Ericson et al., 1997; Briscoe et al., 1999; Sander et al., 2000; Pierani et al., 2001).

The participation of progenitor homeodomain proteins in the conversion of graded Shh signals into all-or-none distinctions in progenitor cell identity depends on cross-

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repressive interactions between selected pairs of class I and II protein (Ericson et al., 1997; Briscoe et al., 2000; Sander et al., 2000; Muhr et al., 2001). In addition, most class I and II proteins have been shown to function directly as transcriptional repressors, through the recruitment of corepressors of the Gro/TLE class (Muhr et al., 2001). These findings have suggested a derepression model of neural patterning which invokes the idea that the patterning activities of individual class I or II proteins are achieved primarily through their ability to repress expression of complementary homeodomain proteins from specific progenitor domains. A central implication of this model is that homeodomain proteins direct progenitor cells to individual neuronal fates by suppressing alternative pathways of differentiation - a view that has strong parallels with proposed mechanisms of lineage restriction during lymphoid differentiation (Nutt et al., 1999; Rolink et al., 1999; Eberhard, et al., 2000).

Much of the evidence that has led to this general outline of ventral neural patterning has emerged from an analysis of members of the *Nkx* gene family. Two closely-related *Nkx* repressor proteins, *Nkx2.2* and *Nkx2.9*, function as class II proteins that specify the identity of V3 neurons (Ericson et al., 1997; Briscoe et al., 1999, 2000). A more distantly related class II repressor protein, *Nkx6.1*, is expressed throughout the ventral third of the neural tube and when ectopically expressed, can direct motor neuron and V2 neuron fates (Briscoe et al., 2000; Sander et al., 2000). These gain-of-function studies are supported by an analysis of mice lacking *Nkx6.1* function, which exhibit a virtually

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complete failure in V2 interneuron generation (Sander et al., 2000). *Nkx6.1* null mice also show a reduction in motor neuron generation at rostral levels of the spinal cord, but at more caudal levels motor neurons are formed in near-normal numbers (Sander et al., 2000). This observation reveals the existence of an *Nkx6.1*-independent program of spinal motor neuron generation, although the molecular basis of this alternative pathway is unclear.

10 A close relative of *Nkx6.1*, termed *Nkx6.2* (also known as *Nkx6B* or *Gtx*), has been identified (Komuro et al., 1993; Lee et al., 2001), and is expressed by neural progenitor cells (Cai et al., 1999). In its alias of *Gtx*, *Nkx6.2* has been suggested to regulate myelin gene expression (Komuro et al., 15 1993), but its possible functions in neural patterning have not been examined. The identification of an *Nkx6* gene pair prompted us to address three poorly resolved aspects of ventral neural patterning. First, do closely related pairs of repressor homeodomain proteins serve distinct or 20 redundant roles in ventral neural patterning? Second, are class I repressor proteins always complemented by a corresponding class II repressor, and if so, is *Nkx6.2* one of the missing class II proteins? Third, to what extent is the generation of spinal motor neurons dependent on the 25 activity of *Nkx6* class proteins?

To address these issues we mapped the profile of expression of *Nkx6.2* and *Nkx6.1* during neural tube development, and analysed mouse *Nkx6* mutants to determine the respective 30 contributions of these two genes to neural patterning. We show that *Nkx6.2*, like *Nkx6.1*, functions as a class II

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repressor homeodomain protein. Our analysis of *Nkx6* mutants further indicates that the duplication of an ancestral *Nkx6* gene has resulted in the expression of two proteins that exert markedly different levels of repressor activity in the ventral neural tube. This differential repressor activity of these two proteins appears to provide both a fail-safe mechanism during motor neuron generation, and the potential for enhanced diversification of ventral interneuron subtypes. Moreover, we find that under conditions of reduced *Nkx6* gene dosage, ventral neuronal subtypes can be generated from progenitor cells that lack the class I or class II proteins normally required for their generation. This finding supports one of the central tenets of the derepression model of ventral neural patterning - that progenitor homeodomain proteins direct particular neuronal fates by actively suppressing cells from adopting alternative fates.

The specification of neuronal fate in the vertebrate central nervous system appears to depend on the profile of transcription factor expression by neural progenitor cells, but the precise roles of such factors in neurogenesis remain poorly understood. A pair of closely-related homeodomain proteins that function as transcriptional repressors, *Nkx6.2* and *Nkx6.1*, are expressed by progenitor cells in overlapping domains of ventral spinal cord. We provide genetic evidence in the mouse that differences in the level of repressor activity of homeodomain proteins underlies the diversification of ventral interneuron subtypes, and provides a fail-safe mechanism during motor neuron generation. We also show that a reduction in *Nkx6* protein

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activity permits V0 neurons to be generated from progenitor cells that lack the homeodomain proteins normally required for their generation. This finding provides direct evidence for a model of neuronal fate specification in which
5 progenitor homeodomain proteins direct specific neuronal fates by actively suppressing the expression of transcription factors that direct alternative fates.

EXPERIMENTAL DETAILS

A. Materials and Methods

5 Generation of *Nkx6.2* mutant mice

Mouse *Nkx6.2* genomic clones were isolated from a 129/Ola mouse genomic library. A targeting construct was constructed by inserting a tau-lacZ/pGKneo cassette into a 5 kb 5' HindIII-NcoI fragment and a 2.7 kb 3' SphI-AccI
10 fragment. The linearized targeting construct was electroporated into E14.1 (129/Ola) ES cells. Cells were selected with G418 and screened by Southern blot analysis using a 200 bp 3' AccI fragment, which detected a 6 kb wild
type band and a 2.9 kb mutant band. Recombinant clones were
15 injected into C57BL/6J blastocysts to generate two chimeric founders, both of which transmitted the mutant allele. Mice homozygous for the mutant alleles were born at Mendelian frequency and survived through adulthood. All experiments involved mice maintained on a C57BL/6 background. The
20 generation and genotyping of *Nkx6.1* mutant mice have been described previously (Sander et al. 2000). Compound *Nkx6* mutant mice were obtained by crossing *Nkx6.2*^{+/lacZ}; *Nkx6.1*^{+/-} double heterozygous mice. Genotyping was performed using Southern blot analysis.

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Chick in ovo electroporation

Mouse *Nkx6.2* was isolated by PCR (Komuro et al., 1993) and chick *Nkx6.2* from a chick spinal cord library (Basler et al., 1993) using mouse *Nkx6.1* and *Nkx6.2* as probes. cDNAs
30 encoding full-length mouse and chick *Nkx6.2* were inserted into a RCASBP(B) retroviral vector and electroporated into

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the neural tube of stage HH (Hamburger and Hamilton, 1953) 10-12 chick embryos (Briscoe et al., 2000). After 24-48h, embryos were fixed and processed for immunohistochemistry.

5 Immunohistochemistry and in situ hybridization
histochemistry

Immunohistochemical localization of proteins was performed as described (Yamada et al., 1993; Briscoe et al., 2000). Guinea-pig antisera were generated against an 11 amino acid
10 N-terminal sequence of mouse *Nkx6.2*. Other antibodies used were rabbit anti-Lim3 (Ericson et al., 1997), mAb Hb9 (Tanabe et al., 1998), rabbit anti-Isl1/2 (Tsuchida et al., 1994), rabbit anti-Chx10 (Ericson et al., 1997), rabbit
anti-En1 (Davis et al., 1991), mAb anti-Evx1/2, rabbit anti-
15 Dbx1, rabbit anti-Dbx2 (Pierani et al., 1999), rabbit anti-*Nkx6.1* (Jørgensen et al., 1999), mAb anti-Pax7 (Ericson et al., 1996), rabbit anti-bgal (Cappel) and goat anti-bgal (Biogeneseis). Images were collected on a Zeiss LSM510 confocal microscope. In situ hybridisation was performed as
20 described (Schaeren-Wiemers and Gerfin-Moser, 1993), using chick probes for *Dbx1*, *Dbx2* (Pierani et al., 1999), *Nkx6.1* (Briscoe et al., 2000) and *Nkx6.2*. A mouse probe for the 5'UTR of *Nkx6.2* comprised 346 bp upstream of the start ATG site. Whole-mount X-gal staining was performed as described
25 (Mombaerts et al., 1996).

B. Results

30 Distinct patterns of *Nkx6.1* and *Nkx6.2* expression in embryonic spinal cord

To examine the roles of *Nkx6* class genes in ventral neuronal

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specification we compared the patterns of expression of *Nkx6.2* and *Nkx6.1* with that of other progenitor homeodomain proteins in the spinal cord of mouse and chick embryos. In the caudal neural tube of the mouse, the expression of *Nkx6.2* was first detected at -e8.5, in a broad ventral domain that largely coincided with that of *Nkx6.1* (Figure 10A). Between e8.5 and e9.5, the expression of *Nkx6.2* was lost from most *Nkx6.1*⁺ cells in the ventral neural tube, although expression persisted in a narrow stripe of cells just dorsal to the limit of *Nkx6.1* expression (Figure 10B, C). At e10.0-e10.5, virtually all, *Nkx6.2*⁺ cells coexpressed *Dbx2* (Figure 10E), and the ventral limit of expression of both *Nkx6.2* and *Dbx2* coincided with the dorsal limit of *Nkx6.1* expression at the p1/p2 domain boundary (Figure 10D, E). *Nkx6.2* was expressed predominantly within the p1 domain, but scattered *Nkx6.2*⁺ cells were detected within the p0 domain - the domain of expression of *Pax7*⁺, *Dbx1*⁺ cells (Figure 10F). Within the p0 domain, however, individual *Nkx6.2*⁺ cells did not coexpress *Dbx1*, although they did express *Dbx2* (Figure 10E-G). Thus, the scattered *Nkx6.2*⁺ cells found at the dorsoventral level of the p0 domain exhibit a p1, rather than p0, progenitor cell identity. Studies in chick have similarly shown that p0 and p1 progenitors are interspersed in the most dorsal domain of the ventral neural tube (Pierani et al., 1999).

In the chick neural tube, as in the mouse, *Nkx6.1* and *Nkx6.2* are initially coexpressed in a broad ventral domain (Cai et al., 1999; data not shown). But in contrast to the mouse, *Nkx6.2* expression persists in ventral progenitor cells, with the consequence that the expression of *Nkx6.2* and *Nkx6.1*

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also overlaps at later developmental stages (Figure 10H, I). Nevertheless, expression of chick *Nkx6.2* is also detected in a thin stripe of cells dorsal to the limit of *Nkx6.1* expression, within the p1 domain (Figure 10H). Thus, in
5 both species, p1 progenitors coexpress *Nkx6.2* and *Dbx2* and exclude *Nkx6.1*.

Nkx6.2 Regulates V0 and V1 Interneuron Fates by Repression of *Dbx1* Expression

10 The establishment and maintenance of progenitor cell domains in the ventral neural tube has been proposed to depend on mutual repressive interactions between complementary pairs of class I and II homeodomain proteins (Briscoe et al.,
2000; Muhr et al., 2001). But class II proteins have been
15 identified for only two of the five known progenitor domain boundaries (the p1/p2 and pMN/p3 boundaries) (Ericson et al., 1997; Briscoe et al., 1999, 2000; Sander et al., 2000). The mutually exclusive pattern of expression of *Nkx6.2* and *Dbx1* within p1 and p0 progenitors led us to consider whether
20 *Nkx6.2* might function as a class II protein that represses *Dbx1* expression, and thus help to establish the identity of p1 progenitor cells and the fate of their *En1*⁺ V1 neuronal progeny.

25 To test this idea, we analysed the profile of expression of class I and II homeodomain proteins in *Nkx6.2* mutant embryos. We inactivated the mouse *Nkx6.2* gene by homologous recombination in embryonic stem (ES) cells. A targeted *Nkx6.2* allele (*Nkx6.2*^{tlz}) was generated by replacing the
30 coding sequence of *Nkx6.2* with a *tauLacZ* cassette (Figure 11A). In the spinal cord of *Nkx6.2*^{+/tlz} embryos analysed at

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el0.5, expression of LacZ and Nkx6.2 coincided within the p1 progenitor domain (see Figure 11E, F). In *Nkx6.2^{tlz/tlz}* embryos, the location of LacZ⁺ cells was also similar to that in *Nkx6.2^{+/tlz}* embryos (Figure 11F, G), but Nkx6.2 protein was not detected (Figure 11G). These data provide evidence that the *Nkx6.2^{tlz}* allele generates a null mutation, and that disruption of the *Nkx6.2* locus does not perturb the normal spatial pattern of expression of this gene.

We did observe, however, that the level of LacZ expression was markedly elevated in *Nkx6.2^{tlz/tlz}*, when compared with *Nkx6.2^{+/tlz}* embryos (Figure 11B-D). An elevation in level of expression of the residual 5' *Nkx6.2* transcript was also detected in *Nkx6.2^{tlz/tlz}* embryos (Figure 11H-J). These observations provide evidence that Nkx6.2 negatively regulates its own expression level within p1 progenitor cells.

We next analysed the pattern of expression of class I and II homeodomain proteins in the spinal cord and caudal hindbrain of *Nkx6.2^{tlz/tlz}* embryos. The domains of expression of the class II proteins Nkx2.2 and Nkx6.1, and of the class I proteins Pax7, Dbx2, Irx3 and Pax6 were similar in *Nkx6.2^{tlz/tlz}*, *Nkx6.2^{+/tlz}*, and wild type embryos (Figure 12B-D, G-I; data not shown). In addition, normal patterns of expression of Dbx2 and Nkx6.1 were detected at the p1/p2 domain boundary (data not shown), showing that establishment of the p1 progenitor domain does not require Nkx6.2 function. However, the level of Dbx2 expression in p1 domain progenitors was increased -two-fold in *Nkx6.2^{tlz/tlz}* mutants (Figure 11K-M), indicating that Nkx6.2 normally limits the

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level of Dbx2 expression in this domain.

We also detected a marked change in the pattern of expression of the p0 progenitor cell marker Dbx1 in *Nkx6.2^{tlz/tlz}* embryos. At caudal hindbrain levels, the number of ventral Dbx1⁺ progenitor cells increased 1.7- fold (Figure 12F), and the domain of Dbx1⁺ cells expanded ventrally, extending through the p1 domain to the dorsal limit of *Nkx6.1* expression (Figure 12H). Moreover, in *Nkx6.2^{tlz/tlz}* embryos all of the ectopic Dbx1⁺ cells found within the p1 domain coexpressed LacZ (Figure 12J). Thus, many progenitors within the p1 domain initiate Dbx1 expression in the absence of *Nkx6.2* function. Nevertheless in *Nkx6.2^{tlz/tlz}* embryos, numerous LacZ⁺ progenitors still lacked Dbx1 expression (Figure 12J), implying the existence of an *Nkx6.2*-independent means of excluding Dbx1 expression from p1 progenitors. The ventral expansion of Dbx1 was most prominent at caudal hindbrain and cervical spinal levels of the neural tube but a similar, albeit less marked, expansion of Dbx1 expression was detected at caudal spinal levels (data not shown; see Figure 15). Taken together, these data imply that within p1 domain progenitors *Nkx6.2* functions as a weak repressor of Dbx2 expression and a more potent repressor of Dbx1 expression.

25

We next analysed the generation of interneuron subtypes in the ventral neural tube. In wild type embryos, Dbx1⁺, Dbx2⁺, *Nkx6.2*⁻ p0 progenitors generate *Evx1/2*⁺ V0 neurons (Pierani et al., 1999; 2001); *Nkx6.2*⁺, Dbx1⁻, Dbx2⁺ p1 progenitors give rise to *En1*⁺ V1 neurons (Burrill et al., 1997; Ericson et al., 1997), and *Nkx6.1*⁺, *Irx3*⁺, p2

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progenitors give rise to Chx10⁺ V2 neurons (Ericson et al., 1997; Briscoe et al., 2000). Dbx1 activity in p0 progenitors is required to promote V0 and suppress V1 neuronal fates (Pierani et al., 2001). The ventral expansion in Dbx1 expression in *Nkx6.2^{tlz/tlz}* embryos therefore led us to examine whether the loss of *Nkx6.2* function leads progenitor cells within the p1 domain to adopt a V0 rather than V1 neuronal fate.

10 In the caudal hindbrain of *Nkx6.2^{tlz/tlz}* embryos examined at e10.5, we detected a ~ two-fold increase in the number of Evx1/2⁺ V0 neurons and the domain of V0 neuronal generation expanded ventrally the normal position of the p1 domain (Figure 12N). Consistent with this, many Evx1/2⁺ neurons

15 coexpressed LacZ (Figure 12P), showing directly that some V0 neurons derive from p1 progenitors in the absence of *Nkx6.2* function. Conversely, the total number of En1⁺ V1 neurons generated in *Nkx6.2^{tlz/tlz}* embryos was reduced by ~50% (Figure 12Q). The dorsoventral position of generation of

20 the remaining En1⁺ V1 neurons was similar in *Nkx6.2^{tlz/tlz}* embryos (Figure 12N), and these neurons expressed LacZ (Figure 12O) showing directly that *Nkx6.2⁺*, *Dbx2⁺* p1 progenitor cells generate V1 neurons. The total number of neurons generated from p1 domain progenitors, defined by

25 *Cyn1*, *TuJ1* and *Lim1/2* expression was similar in *Nkx6.2^{tlz/tlz}* and *Nkx6.2^{+/tlz}* embryos examined at e10.5 (data not shown). In addition, the number of TUNEL⁺ cells was similar in *Nkx6.2^{tlz/tlz}* and *Nkx6.2^{+/tlz}* embryos (data not shown). Chx10⁺ V2 neurons and HB9⁺, *Isl1/2⁺* motor neurons were present in

30 normal numbers and positions in *Nkx6.2^{tlz/tlz}* embryos (Figure 14; data not shown). Together, these findings show that the

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activity of *Nkx6.2* within p1 progenitors promotes V1 neuronal generation and helps to suppress the generation of V0 neurons, a finding consistent with the proposed role of *Nkx6.2* in repressing *Dbx1* expression from p1 progenitors.

5

Repression of *Nkx6.2* by *Nkx6.1* underlies *Nkx6* gene redundancy in spinal motor neuron generation

We next addressed the respective contributions of *Nkx6.1* and *Nkx6.2* to motor neuron and V2 neuron generation. In the ventral neural tube, p2 and PMN progenitors express *Nkx6.1* and give rise to V2 neurons and motor neurons respectively. Ectopic expression of *Nkx6.1* is sufficient to induce motor neurons and V2 interneurons in dorsal regions of the neural tube, and in *Nkx6.1* mutant mice V2 neurons are eliminated (Briscoe et al., 2000; Sander et al., 2000). Nevertheless, there is only a partial reduction in motor neuron generation in *Nkx6.1* mutants (Sander et al., 2000), revealing the existence of an *Nkx6.1*-independent pathway of motor neuron generation. *Nkx6.2* does not normally contribute to motor neuron specification in the mouse, since its expression is extinguished from ventral progenitors well before the appearance of post-mitotic motor neurons (Figure 10A-C), and there is no change in the number of motor neurons generated in *Nkx6.2*^{tlz/tlz} embryos (see Figure 14G).

25

Three lines of evidence, however, led us to consider a cryptic role for *Nkx6.2* in motor neuron generation. First, *Nkx6.2* and *Dbx2* share the same ventral limit of expression at the p1/p2 domain boundary, and the expression of *Dbx2* is repressed by *Nkx6.1* (Briscoe et al., 2000; Sander et al., 2000). Second, *Nkx6.2* negatively regulates its own

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expression level within p1 domain progenitors (Figure 11D, G, J). Third, *Nkx6.1* and *Nkx6.2* possess similar Gro/TLE recruitment activities and DNA target site binding specificities (Muhr et al., 2001). We reasoned therefore
5 that under conditions in which *Nkx6.1* activity is reduced or eliminated, *Nkx6.2* expression might be derepressed in p2 and pMN progenitors.

In support of this idea, in *Nkx6.1*^{+/-} embryos examined at e10.5 we detected a marked increase in the number of *Nkx6.2*⁺
10 cells within the p2 and pMN domains (Figure 13B). And in *Nkx6.1*^{-/-} embryos, expression of *Nkx6.2* was detected in virtually all progenitor cells within the p2 and pMN domains (Figure 13C). Indeed, in *Nkx6.1*^{-/-} embryos, the level of *Nkx6.2* expression in the nuclei of progenitor cells within
15 the p2 and pMN domains was 1.9-fold greater than that in progenitor cells located within the p1 domain (Figure 13C; data not shown). Together, these data show that *Nkx6.1* activity normally represses *Nkx6.2* expression from p2 and pMN progenitors in the mouse embryo.

20 In turn, these findings raised the possibility that in *Nkx6.1*^{-/-} embryos, the derepression of *Nkx6.2* expression substitutes for the loss of *Nkx6.1* during motor neuron generation. If this is the case, *Nkx6.2* would be predicted
25 to mimic the ability of *Nkx6.1* to induce motor neurons in vivo. Expression of chick or mouse *Nkx6.2* in the neural tube of HH stage 10-12 chick embryos repressed *Dbx2* and *Dbx1* expression (Figure 13D-F), and induced ectopic motor neuron differentiation (Figure 13G-I, L-N) with an efficacy similar
30 to that of *Nkx6.1* (Briscoe et al., 2000). These data show that *Nkx6.2* can induce ectopic motor neurons when expressed

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at high levels in the dorsal neural tube, supporting the idea that both *Nkx6* proteins can exert similar patterning activities in vivo (Figure 13D-O; Briscoe et al., 2000). In addition, misexpression of *Nkx6.2* in the p0 and p1 progenitor domains suppressed the generation of *Evx1/2*⁺ V0 and *En1*⁺ V1 neurons and promoted the generation of *Chx10*⁺ V2 neurons (Figure 13J, K, O, P). Thus, a high level of expression of *Nkx6.2* is not compatible with the generation of either V0 or V1 neurons (Figure 13O, P).

10

Based on these findings, we examined whether *Nkx6.2* has a role in motor neuron generation in *Nkx6.1* mutant mice by testing the impact of removing *Nkx6.2* as well as *Nkx6.1* on the generation of spinal motor neurons. In *Nkx6.2*^{tlz/tlz} embryos there was no change in the number of motor neurons generated at any level of the spinal cord or hindbrain (Figure 14G,N,O; data not shown). In *Nkx6.1*^{-/-} mutants, the number of spinal motor neurons was reduced by ~60% at cervical levels, but by only 25% at lumbar levels (Figure 14H,N,O, Sander et al., 2000). In *Nkx6.1*^{-/-}; *Nkx6.2*^{+/tlz} embryos, motor neuron generation was reduced to ~25% of controls at both cervical and lumbar levels (Figure 14I,N,O; data not shown). In *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tlz} embryos, the generation of motor neurons was reduced to <10% of wild type numbers, at all levels of the spinal cord (Figure 14J). In these *Nkx6* double mutant embryos, residual motor neurons were detected at e10.0, and no further increase in motor neuron number was evident at e12 (Figure 14M, P; data not shown). Since there was no increase in apoptotic cell death in the ventral neural tube over this period (data not shown), we infer that the few spinal motor neurons present

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in *Nkx6* double mutants are generated prior to e10. Together, these findings demonstrate that *Nkx6.2* substitutes for the loss of *Nkx6.1* in spinal motor neuron generation, and reveal a link between *Nkx6* gene dosage and the incidence of motor neuron generation.

A Dissociation in Neuronal Fate and Progenitor Cell Identity in *Nkx6* Mutant Mice

We next examined whether a reduction in *Nkx6* gene dosage results in ectopic Dbx protein expression and V1 and V0 neuron generation in the p2 and pMN domains of the ventral spinal cord.

En1⁺ V1 neurons are normally generated from Dbx2⁺, Dbx1⁻ p1 progenitor cells, and we therefore analysed the relationship between Dbx2 expression and En1⁺ V1 neuronal generation in *Nkx6.1* and *Nkx6.2* compound mutants. As reported previously (Sander et al., 2000), in *Nkx6.1*^{-/-} embryos examined at e10.5, ectopic ventral expression of Dbx2 was detected at high levels in the p2 and p3 domains, although cells in the pMN expressed only very low levels of Dbx2 (Figure 15H; see Sander et al., 2000). Moreover, in *Nkx6.1*^{-/-} embryos, ectopic En1⁺ neurons were generated in the p2 and pMN domains of the ventral neural tube (Figure 15R). In *Nkx6.1*^{-/-}; *Nkx6.2*^{+/-^{tlz}} embryos, Dbx2 expression was detected at intermediate levels in the pMN domain (Figure 15I), and in *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tl1} double mutant embryos, Dbx2 was detected at uniformly high levels in the p2 and pMN domains (Figure 15J). Strikingly, in these *Nkx6.1* and *Nkx6.2* compound mutant backgrounds, and despite the enhanced ectopic expression of Dbx2, the number of ectopic ventral

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En1⁺ V1 neurons was reduced rather than increased, when compared with the number generated in *Nkx6.1* single mutants (Figure 15R, T).

5 Since *Evx1*⁺ V0 neurons are normally generated from *Dbx1*⁺, *Dbx2*⁺ p0 progenitors, we examined whether the reduction in ectopic ventral En1⁺ V1 neuron generation at low *Nkx6* gene dosage might reflect a change in the pattern of expression of *Dbx1*, and the ectopic generation of V0 neurons.
10 Consistent with this idea, in *Nkx6.1*^{-/-}; *Nkx6.2*^{tlz/tlz} mutants, scattered *Dbx1*⁺ cells were detected in the p2, pMN and p3 domains (Figure 15O), and ectopic ventral *Evx1/2*⁺ V0 neurons were detected throughout the ventral neural tube (Figure 15T, Z). Thus, in *Nkx6* double mutants, the loss of V1
15 neurons is associated with the ectopic ventral expression of *Dbx1* and the generation of ectopic V0 neurons.

But in *Nkx6.1* single and *Nkx6.1*^{-/-} ; *Nkx6.2*^{+/tlz} compound mutant backgrounds, the normal link between expression of
20 *Dbx1* in progenitor cells and the generation of *Evx1/2*⁺ V0 neurons was severed. In both these *Nkx6* compound mutants backgrounds, the domain of expression of *Dbx1* was unchanged (Figure 15M, N): a result that can be accounted for by the maintained expression of *Nkx6.2* within the p1 domain, and
25 the deregulated expression of *Nkx6.2* within the p2 and pMN domains. Nevertheless, *Evx1/2*⁺ V0 neurons were generated from progenitor cells in the position of p2 and pMN domains, (Figure 15R, S, X, Y).

30 We next considered whether these ectopic V0 neurons were generated from the position of the p2 and pMN domains, or

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whether they simply migrated ventrally from a more dorsal position of origin. Ectopic ventral *Evx1/2*⁺ V0 neurons were detected as early as e10.0 (Figure 16B), and many of them coexpressed LacZ (Figure 16C, D), providing evidence that many of these neurons derive from progenitor cells within the position of the p2 and pMN domains. The finding that *Evx1/2*⁺ V0 neurons are generated from the pMN domain in *Nkx6.1*^{-/-}; *Nkx6.2*^{+/-LacZ} embryos is especially significant, since these progenitors express negligible levels of *Dbx2* (Figure 16E, 17), arguing against the possibility that *Dbx2* expression compensates for the absence of *Dbx1* during ectopic V0 neuronal generation. These results therefore provide evidence that even though *Dbx1* activity is normally required for the generation of V0 neurons (Pierani et al., 2001), under conditions in which *Nkx6* gene dosage is markedly reduced, V0 neurons can be generated from progenitor cells that lack *Dbx1* expression.

Nevertheless, the pattern of ventral neurogenesis observed in *Nkx6.1*^{-/-}; *Nkx6.2*^{+/-LacZ} mutants indicated that residual *Isl1/2*⁺, *HB9*⁺ neurons and ectopic *Evx1*⁺ neurons were each generated from progenitors located in the position of the pMN domain. This observation raised the question of whether these two neuronal populations are, in fact, distinct. Strikingly, we found that in this compound *Nkx6* mutant background, many of the residual *Isl1/2*⁺, *HB9*⁺ neurons transiently expressed *Evx1* (Figure 16H, I). Thus, under conditions of reduced *Nkx6* gene dosage, progenitor cells at the position of the pMN domain initially generate neurons with a hybrid motor neuron/V0 neuron identity.

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c. Discussion

The patterning of cell types in the ventral neural tube depends on the actions of a set of homeodomain proteins expressed by neural progenitor cells. Duplication of many of these genes has resulted in the overlapping neural expression of pairs of closely-related homeodomain proteins, and raises the question of whether these proteins have distinct or redundant roles during ventral neurogenesis. We have used genetic approaches in mouse to examine the respective contributions of one such homeodomain protein pair, Nkx6.1 and Nkx6.2, in ventral neural patterning. Our results imply that the duplication of an ancestral Nkx6 gene confers both redundant and distinct roles for Nkx6.1 and Nkx6.2 in ventral neuronal patterning. We discuss below how the specificity and efficacy of Nkx6-mediated transcriptional repression underlies the overlapping divergent patterning activities of the two proteins.

20 Redundant Activities of Nkx6 Proteins in Motor Neuron and V0 Neuron Generation

Our genetic studies in mice indicate that Nkx6.1 and Nkx6.2 have qualitatively similar activities in promoting the generation of motor neurons and in suppressing the generation of V0 neurons. How are these overlapping patterning activities achieved, given the distinct profiles of expression of these two genes?

Nkx6.1 has been shown to have a role in motor neuron generation (Sander et al., 2000), but the finding that large numbers of motor neurons are generated at caudal levels of

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the spinal cord in *Nkx6.1* mutant mice, points to the existence of an *Nkx6.1*-independent pathway of motor neuron generation. At face value, *Nkx6.2* would appear a poor candidate as a mediator of the *Nkx6.1*-independent pathway of motor neuron specification, since it is not expressed by motor neuron progenitors, nor is motor neuron generation impaired in *Nkx6.2* mutant mice. Nevertheless, the activity of *Nkx6.2* is responsible for the efficient generation of spinal motor neurons in *Nkx6.1* mutants. The basis of this redundant function resides in the derepression of *Nkx6.2* expression in motor neuron progenitors in *Nkx6.1* mutant mice. Strikingly, *Nkx6.2* is even derepressed in *Nkx6.1*^{+/-} embryos, whereas there is no change in the patterns of expression of *Dbx2* and other homeodomain proteins implicated in the repression of motor neuron generation. The propensity for *Nkx6.2* derepression thus appears to establish a "fail-safe" mechanism that ensures that the net level of *Nkx6* protein activity is maintained in motor neuron progenitors under conditions in which *Nkx6.1* levels decrease. A similar "fail-safe" regulatory mechanism may operate with other *Nkx* protein pairs. During pharyngeal pouch development, for example, the loss of *Nkx2.6* expression appears to be compensated for by the up-regulation of *Nkx2.5* (Tanaka et al., 2000).

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The finding that *Nkx6.2* is derepressed in the absence of *Nkx6.1* function also offers a potential explanation for the divergent patterns of expression of *Nkx6.2* in the ventral neural tube of mouse and chick embryos. We infer that the chick *Nkx6.2* gene is not subject to repression by *Nkx6.1*, permitting its persistent expression in p3, PMN and p2

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domain progenitor cells. Thus, in chick, the overlapping functions of Nkx6.1 and Nkx6.2 in motor neuron generation are associated with the coexpression of both genes by motor neuron progenitors, whereas in the mouse, Nkx6.2 activity is held in reserve, through its repression by Nkx6.1.

Nkx6.1 and Nkx6.2 also have an equivalent inhibitory influence on the generation of V0 neurons, albeit through activities exerted in different progenitor domains. In p1 progenitors, the repression of p0 identity and V0 neuron fate is accomplished by Nkx6.2. But ventral to the p1/p2 domain boundary it is Nkx6.1 that prevents Dbx1 expression and V0 neuronal generation. Thus, Nkx6.1 is a potent repressor of Dbx1 expression, despite the fact that these two proteins lack a common progenitor domain boundary. The repression of genes that are normally positioned in spatially distinct domains has been observed with other class I and II proteins (Sander et al., 2000). This feature of neural patterning also parallels the activities of gap proteins in anteroposterior patterning of the *Drosophila* embryo, where the repressive activities of individual gap proteins are frequently exerted on target genes with which they lack a common boundary (Kraut and Levine, 1991; Stanojevic et al., 1991).

Distinct Functions of Nkx6.1 and Nkx6.2 in Ventral Interneuron Generation

We now turn to the question of how Nkx6.1 and Nkx6.2 can exert distinct roles in interneuron generation, given the similarities of the two proteins in DNA target site specificity (Jorgensen et al., 1999; Muhr et al., 2001), and

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their overlapping functions in the patterning of motor neurons and V0 neurons.

One factor that contributes to the opponent influence of Nkx6.1 and Nkx6.2 on the specification of V1 interneuron fate is a distinction in the dorsal limit of expression of the two proteins in the neural tube, presumably a reflection of differences in the regulation of expression the two proteins by graded Shh signalling. Nkx6.1 expression stops at the p1/p2 domain boundary. And within the p2 domain, Nkx6.1 suppresses p1 progenitor identity through repression of Dbx2 and Nkx6.2 expression, in this way ensuring the generation of Chx10⁺ V2 neurons. Nkx6.2, in contrast, occupies the p1 domain, where it is coexpressed with Dbx2. In p1 domain cells, Nkx6.2 promotes the generation of En1⁺ V1 neurons by repressing the expression of Dbx1 and Evx1, determinants of V0 neuronal fate (Pierani et al., 2001; Moran-Rivard et al., 2001). Nevertheless, only a fraction of p1 progenitors initiate Dbx1 expression and acquire V0 neuron fate in the absence of Nkx6.2 function, raising the possibility that Dbx2 may also have a role in repressing Dbx1 expression within p1 progenitors (see Pierani et al., 1999).

The second major factor that underlies the opponent activities of Nkx6.1 and Nkx6.2 in V1 interneuron specification appears to be a difference in the potency with which the two Nkx6 proteins repress a common set of target genes. This view is supported by several observations. Nkx6.1 completely represses Nkx6.2, whereas Nkx6.2 exerts an incomplete negative regulation of its own expression in

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p1 domain progenitors. Thus, Nkx6.1 is evidently a better repressor of Nkx6.2 than is Nkx6.2 itself. Similarly, Nkx6.2 is coexpressed with Dbx2 in p1 domain progenitors, whereas Nkx6.1 excludes Dbx2 from p2 domain progenitors, indicating that Nkx6.1 also is a more effective repressor of Dbx2 expression than is Nkx6.2. Consistent with this view, Nkx6.2 fails to repress Dbx2 expression completely from ventral progenitors in Nkx6.1 mutants. The fact that Nkx6.2 is only a weak repressor of Dbx2 is critical for the formation of the p1 domain, since the maintained expression of Dbx2 in these cells ensures the exclusion of Nkx6.1 expression (Briscoe et al., 2000).

Our results do not resolve why Nkx6.2 is a weaker repressor than Nkx6.1 in vivo. Differences in the primary structure of Nkx6.2 and Nkx6.1 (Cai et al., 1999; Muhr et al., 2001) could result in an intrinsically lower repressor activity of Nkx6.2, when compared with that of Nkx6.1. But our findings are also consistent with the possibility that the two Nkx6 proteins have inherently similar repressor activities, and that the Nkx6.2 protein is merely expressed at a lower level. Indeed within p1 progenitors, the level of Nkx6.2 expression is clearly subject to tight regulation, with significant consequences for neuronal specification. The selective expression of Nkx6.2 in p1 progenitors, coupled with its weak negative autoregulatory activity, ensures a level of Nkx6 activity that is low enough to permit Dbx2 expression but is still sufficient to repress Dbx1 expression, thus promoting the generation of V1 neurons.

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Our findings therefore reveal that a gradient of extracellular Shh signalling is translated intracellularly into stepwise differences in the level of Nkx6 activity along the ventral-to-dorsal axis of the neural tube. Moreover, the different Nkx6 protein activity levels within ventral progenitor cells are a critical determinant of ventral neuronal fate. Cells that express low or negligible levels of Nkx6 activity (p0 progenitors) are directed to a V0 neuronal fate, cells that express an intermediate Nkx6 activity level (p1 progenitors) are directed to a V1 fate, and cells that express a high Nkx6 activity level (pMN and p2 progenitors) are directed to a motor neuron or V2 fate (Figure 17).

15 Nkx6 Repressor Function and Neuronal Patterning by Derepression

The finding that many progenitor homeodomain proteins exert mutual-cross repressive interactions has led to a model of spinal neuronal patterning based on transcriptional derepression (Muhr et al., 2001). Similar cross-repressive interactions may establish regional progenitor domains in more rostral regions of the developing CNS (Toresson et al., 2000; Yun et al., 2001). A premise of this model is that transcriptional repression is exerted at two sequential steps in neurogenesis. One repressive step operates at the level of the progenitor homeodomain protein themselves, but a second repressive step is exerted on neuronal subtype determinant factors that have a downstream role in directing neuronal subtype fates (Briscoe et al., 2000; Muhr et al., 2001).

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Our analysis of *Nkx6* compound mutant mice provides direct support for this two-step repression model, and in addition indicates that progenitor homeodomain proteins and neuronal subtype determinants differ in their sensitivity to repression by the same class II protein. Normally, the functions of *Dbx1* and *Evx1* are required sequentially during the generation of V0 neurons (Pierani et al., 2001; Moran-Rivard et al., 2001). In *Nkx6.1*^{-/-}; *Nkx6.2*^{+/-} mutants, however, the generation of *Evx1*⁺ V0 neurons occurs in the absence of expression of *Dbx1* by neural progenitor cells. *Dbx1* expression is therefore dispensable for V0 neuron generation under conditions of reduced *Nkx6* gene dosage. From these results, we infer that the net level of *Nkx6* protein activity in ventral progenitor cells is still above threshold for repression of *Dbx1* expression, but is below the level required for repression of *Evx1* expression. These data therefore support the idea that *Nkx6* proteins normally inhibit V0 neuronal fate by repressing the class I progenitor homeodomain protein *Dbx1*, and independently by repressing expression of the V0 neuronal subtype determinant *Evx1*.

A differential sensitivity of progenitor homeodomain proteins and neural subtype determinants to repression appears therefore to underlie the dissociation of progenitor cell identity and neuronal fate observed in *Nkx6* mutants. Such two-tiered repression is, in principle, necessary to specify neuronal fate through transcriptional derepression. In the case of *Nkx6.1*, for example, repression of *Dbx1* and *Dbx2* (and possible other unidentified repressors) should be sufficient to derepress motor neuron subtype determinants such as *MNR2* and *Lim3* in pMN progenitors. But, unless *Nkx6.1*

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also represses the expression of V0 determinants, *Evx1* expression would also be initiated in differentiating motor neurons, resulting in a hybrid neuronal phenotype. Indeed, under conditions in which *Nkx6* gene dosage is reduced or eliminated, some of the neurons generated from the position of the pMN domain do transiently express a hybrid motor neuron/V0 neuron phenotype.

The derepression model also invokes the idea that a major role of *Nkx6* class proteins is to exclude the expression of *Dbx2* and other proteins that inhibit motor neuron generation. This view offers a potential explanation of why a few residual motor neurons are generated in *Nkx6* double mutants. We find that in the absence of *Nkx6* gene function, residual motor neurons are generated only at early developmental stages, suggesting that progenitor cells within the position of the pMN domain have committed to a motor neuron fate prior to the onset of the deregulated ventral expression of *Dbx2* and other motor neuron repressors. We note that a third *Nkx6*-like gene exists in the mouse, but this gene is not expressed in the spinal cord of wild type or *Nkx6* mutant embryos (E. Anderson and J. Ericson, unpublished data), and thus its activity appears not to account for the residual motor neurons generated in *Nkx6* double mutants. Importantly, the detection of residual motor neurons in *Nkx6* double mutants also provides evidence that *Nkx6* proteins do not have essential functions as transcriptional activators during motor neuron specification, further supporting their critical role as repressors.

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Finally, the present studies and earlier work on neurogenesis in the ventral spinal cord (Ericson et al., 1996; Thaler et al., 1999; Arber et al., 1999; Sander et al., 2000) have provided evidence that newly-generated
5 neurons can sometimes express mixed molecular identities. These observations raise the possibility that repressive interactions that select or consolidate individual neuronal identities are not restricted to progenitor cells. Consistent with this view, *Evx1* is required to establish V0
10 and repress V1 neuronal identity through an action in post-mitotic neurons (Moran-Rivard et al., 2001), although it remains unclear whether *Evx1* itself functions in this context as an activator or repressor. Similarly, the
homeodomain protein HB9 has been implicated in the
15 consolidation of motor neuron identity, through repression of V2 neuronal subtype genes (Arber et al., 1999; Thaler et al., 1999). HB9 possesses an eh-1 Gro/TLE recruitment domain (Muhr et al., 2001), suggesting that HB9 controls the
identity of post-mitotic motor neurons through a direct
20 action as a transcriptional repressor. The consolidation of neuronal subtype identity in the spinal cord may therefore depend on transcriptional repressive interactions within both progenitor cells and post-mitotic neurons.

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-77-

What is claimed is:

1. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.
2. The method of claim 1, wherein the nucleic acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell.
3. The method of claim 1, wherein the nucleic acid is introduced by transfection or transduction.
4. The method of claim 1, wherein the ventral neuron is a motor neuron, a V2 interneuron or a V3 interneuron.
5. A method of diagnosing a motor neuron degenerative disease in a subject which comprises:
 - a) obtaining a nucleic acid sample from the subject;
 - b) sequencing the nucleic acid sample; and
 - c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid

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sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

5

6. The method of claim 5, wherein the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

10

7. A method of diagnosing a motor neuron degenerative disease in a subject which comprises:

- a) obtaining a nucleic acid sample from the subject;
- b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes;
- c) separating the resulting nucleic acid fragments by size fractionation;
- d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands;

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- d) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and
- 5 f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands
- 10 of step (d) to the control sample indicates that the subject has the motor neuron degenerative disease.
- 15

8. The method of claim 7, wherein the nucleic acid is DNA.
- 20 9. The method of claim 7, wherein the nucleic acid is RNA.
10. The method of claim 7, wherein the size fractionation in step (c) is effected by a polyacrylamide or agarose gel.
- 25 11. The method of claim 7, wherein the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label.
- 30

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12. The method of claim 7, wherein the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.
- 5 13. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.2 protein in the stem cell so as to thereby convert the stem cell into
10 the ventral neuron.
14. The method of claim 13, wherein the nucleic acid introduced into the stem cell incorporates into
the chromosomal DNA of the stem cell.
-
- 15 15. The method of claim 13, wherein the nucleic acid is introduced by transfection or transduction.
16. The method of claim 13, wherein the ventral neuron
20 is a motor neuron.
17. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain
25 transcription factor Nkx6.1 in the stem cell so as to thereby convert the stem cell into the ventral neuron.
18. The method of claim 17, wherein the ventral neuron
30 is a motor neuron, a V2 interneuron or a V3 interneuron.

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19. A method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a polypeptide which expresses homeodomain transcription factor Nkx6.2 in the stem cell so as to thereby convert the stem cell into the ventral neuron.

20. The method of claim 19, wherein the ventral neuron is a motor neuron.

21. A method of diagnosing a neurodegenerative disease in a subject which comprises:

- a) obtaining a suitable sample from the subject;
- b) extracting nucleic acid from the suitable sample;
- c) contacting the resulting nucleic acid with a nucleic acid probe, which nucleic acid probe (i) is capable of hybridizing with the nucleic acid of Nkx6.1 or Nkx6.2 and (ii) is labeled with a detectable marker;
- d) removing unbound labeled nucleic acid probe; and
- e) detecting the presence of labeled nucleic acid, wherein the presence of labeled nucleic acid indicates that the subject is afflicted with a chronic neurodegenerative disease, thereby diagnosing a chronic neurodegenerative disease in the subject.

22. The method of claim 21, wherein the suitable sample is spinal fluid.

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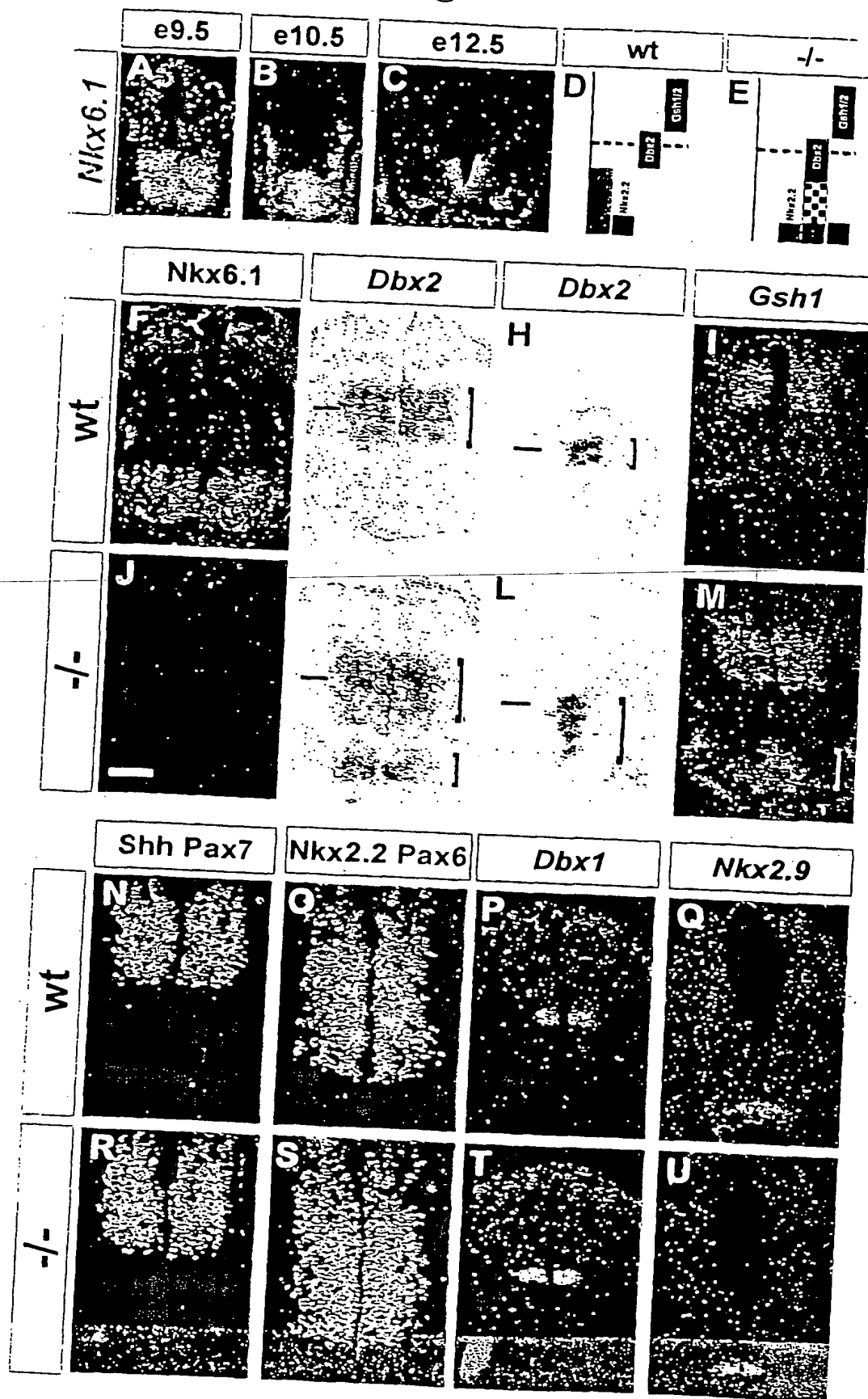
23. The method of claim 21, wherein the nucleic acid
is DNA.

24. The method of claim 21, wherein the nucleic acid
is RNA.

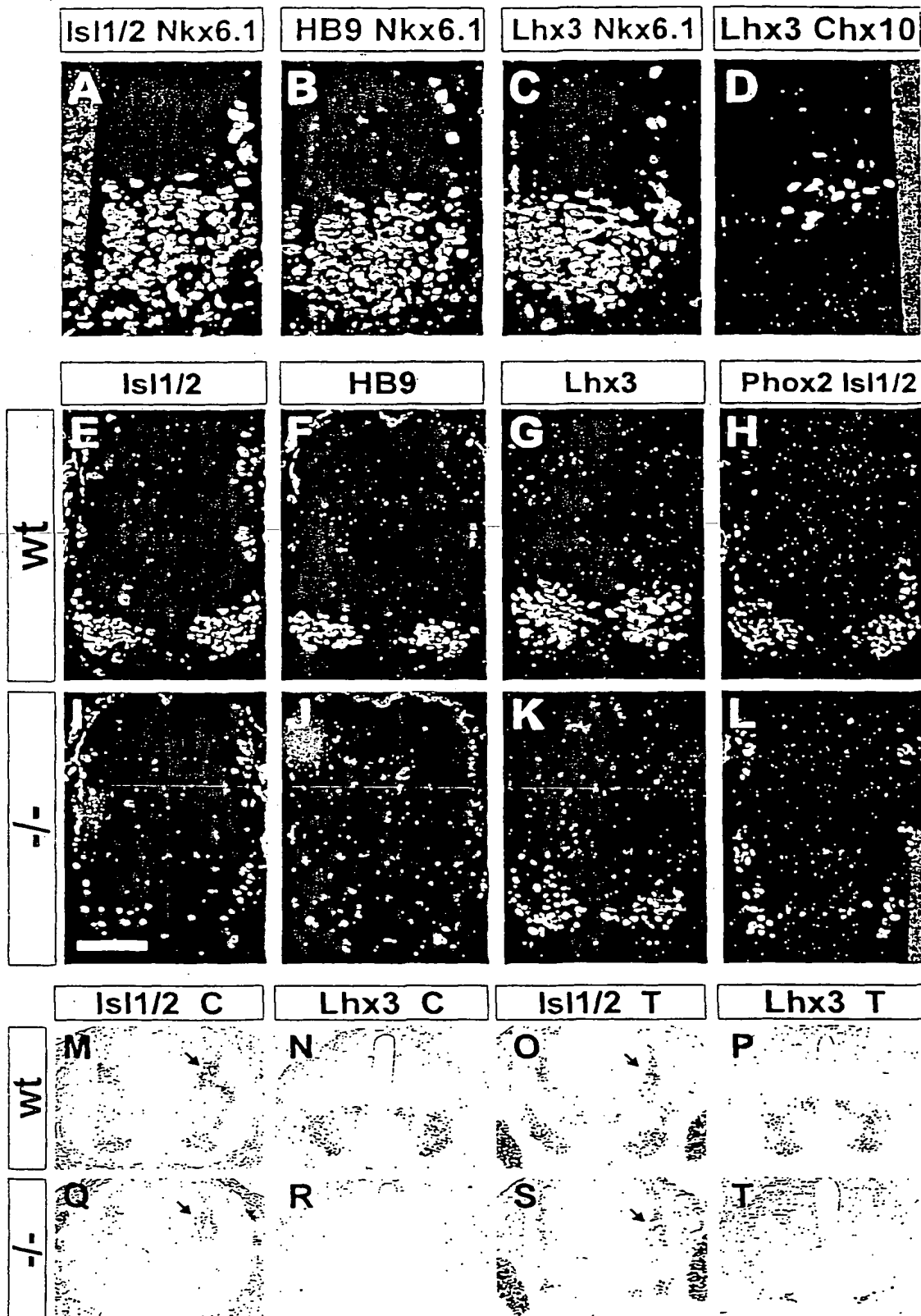
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Figure 1

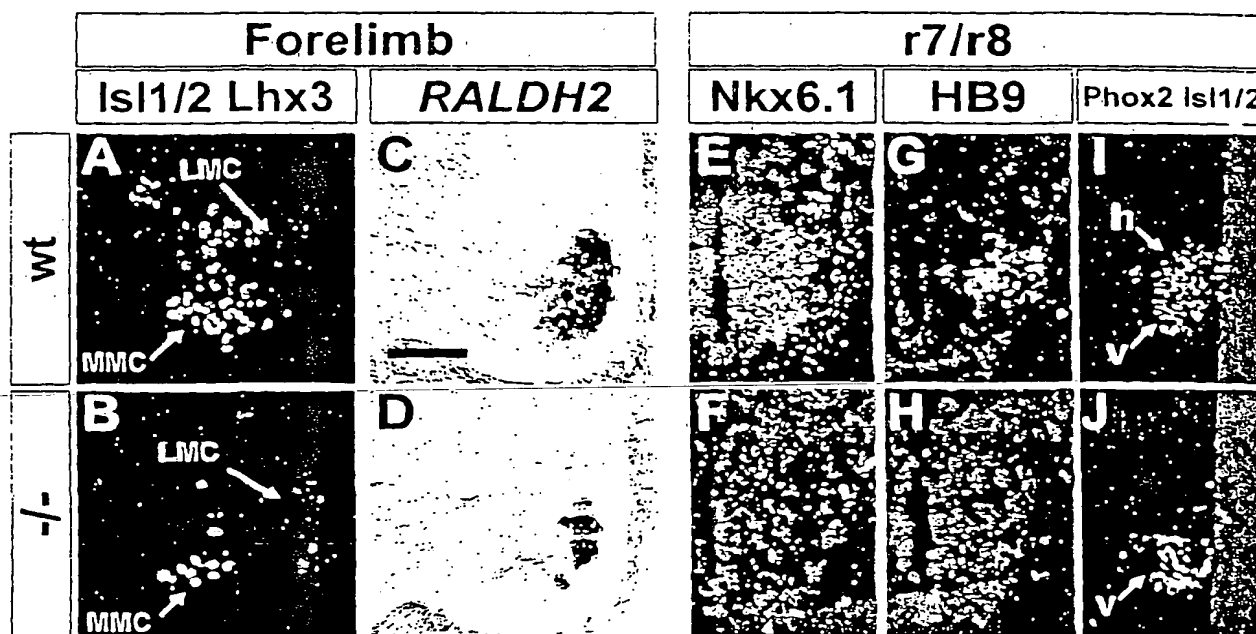


2/22 Figure 2



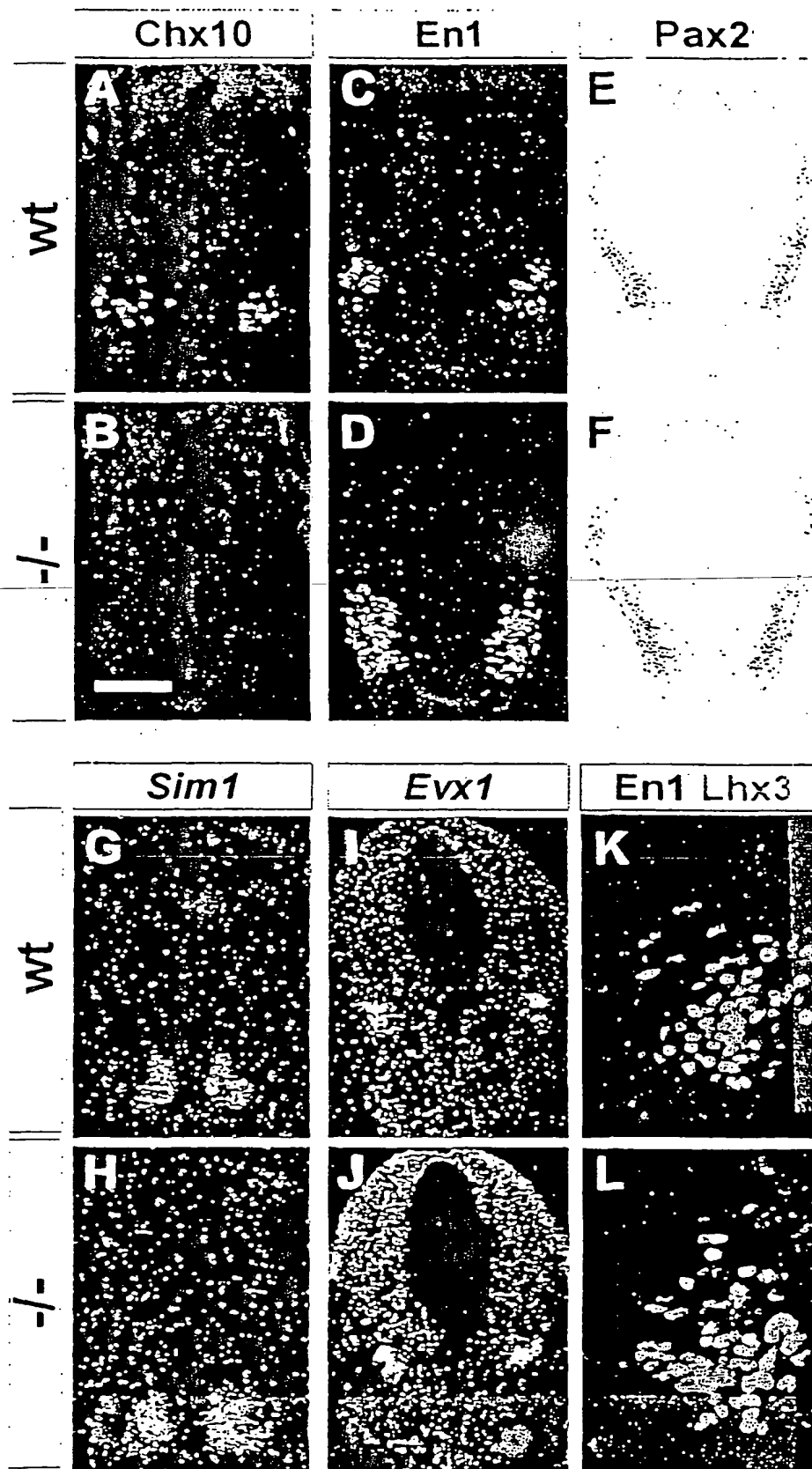
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Figure 3



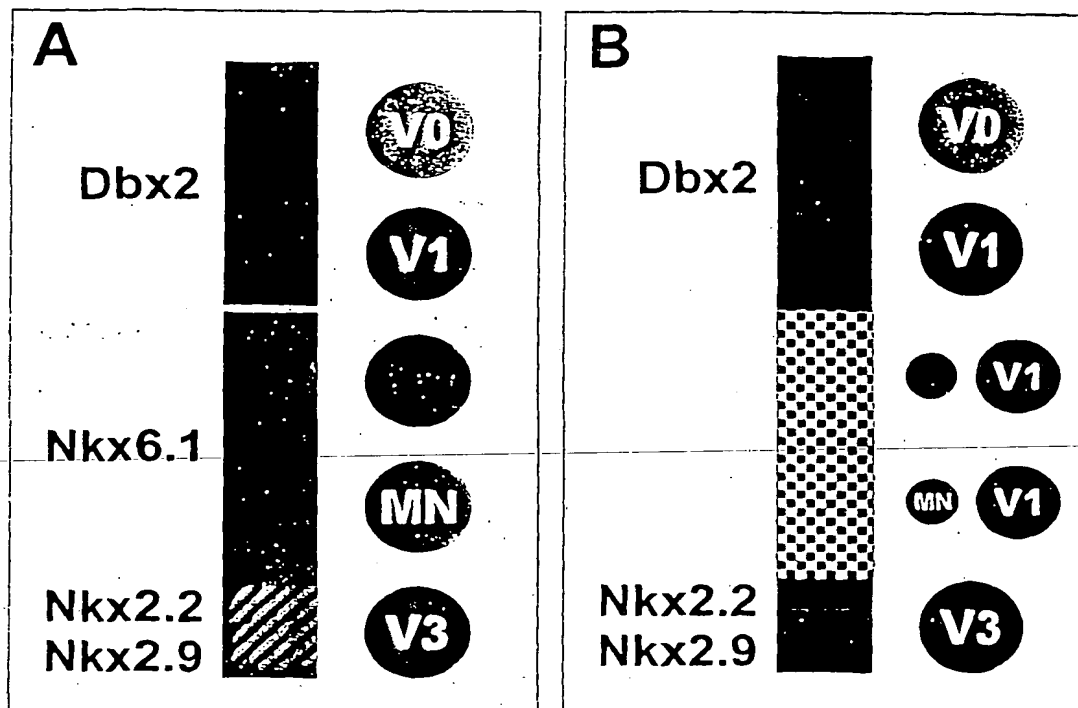
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Figure 4



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Figure 5



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Figure 6

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1  mlavgamegt rqsafllssp plaalhsmæ mktplypaay pplpagppss sssssssssp
61 spplgthnpg glkppatggl sslgsppqql saatphginn ilsrpmpva sgaalpsasp
121 sgssssssss asassasaaa aaaaaaaaaæ sspagllagl prfsslspss pppglyfssp
181 aaavaavgry pkplaelpgr tpifwpgvmq sppwrdarla ctpggsill dkdgkrkhtr
241 ptfsgqqifa lektfeqtky lagperarla yslgmtesqv kvwfgnrrtk wrkkhaaema
301 takkkqdsæ erlkgasene eedddynkpl dpnsddekitt qlkkkhksss ggggglllha
361 sepeesss
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Figure 7

```
1  cgtgggatgt  tagcggtggg  ggcaatggag  ggcacccggc  agagcgcatt  cctgctcagc
61  agccctcccc  tggccgccct  gcacagcatg  gccgagatga  agaccccgct  gtaccctgcc
121  gcgtatcccc  cgtgcctgc  cggccccccc  tcctcctcgt  cctcgtcgtc  gtcctcctcg
181  tcgccctccc  cgcctctggg  caccacaaac  ccaggcggcc  tgaagcccc  ggccacgggg
241  gggctctcat  ccctcggcag  cccccgcag  cagctctcgg  ccgccacccc  acacggcate
301  aacaatatcc  tgagccggcc  ctccatgccc  gtggcctcgg  gggccgccct  gccctccgcc
361  tcgccctccg  gttcctcctc  ctctcttcc  tcgtccgcct  ctgcctcctc  cgcctctgcc
421  gccgccgcgg  ctgctgccgc  ggccgcagcc  gccgcctcat  ccccgggcgg  gctgctggcc
481  ggactgccac  gctttagcag  cctgagcccg  ccgccgccgc  cgcccgggct  ctacttcage
541  cccagcgccg  cggccgtggc  cgcctggggc  cggtacccc  agccgctggc  tgagctgcct
601  ggccggacgc  ccatcttctg  gcccggagtg  atgcagagcc  cgccctggag  ggacgcacgc
661  ctggcctgta  cccctcgtga  gt
```

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Figure 8

```
1  tcacagatca aggatccatt ttgttggaca aagacgggaa gagaaaacac acgagaccca
61  ctttttcogg acagcagatc ttcgccctgg agaagacttt cgaacaaaca aaatacttgg
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181 tgagt
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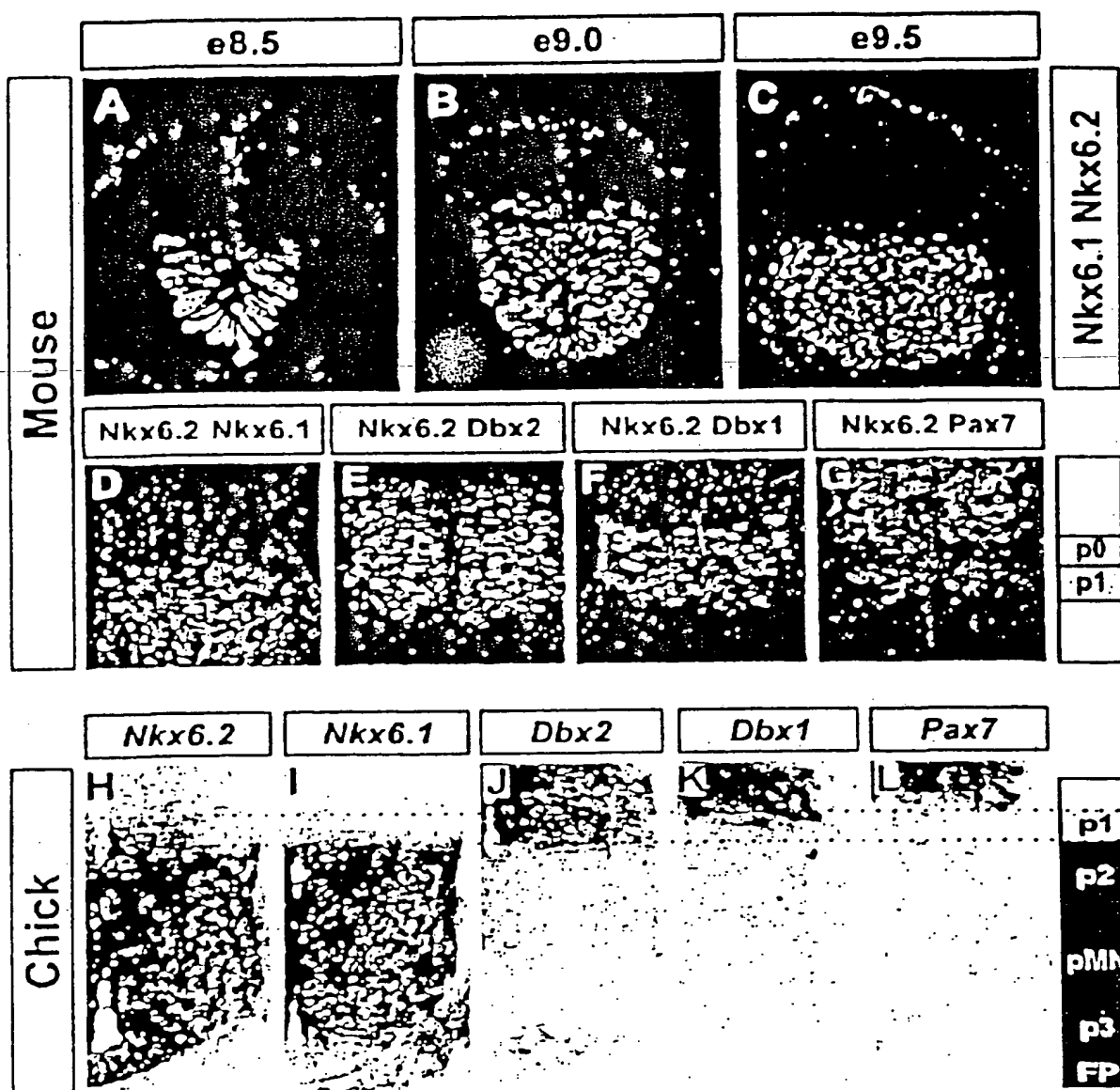
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Figure 9

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1 cctcaggtct ggttcagaa ccgccggacc aagtggagga agaagcacgc tgccgagatg
61 gccacggcca agaagaagca ggactcggag acagagcgcc tcaagggggc ctcggagaaac
121 gaggaagagg acgacgacta caataagcct ctggatccca actcggacga cgagaaaatc
181 acgcagctgt tgaagaagca caagtccagc agcggcggcg gcggcggcct cctactgcac
241 gcgtccgagc cggagagctc atcctgaacg ccg
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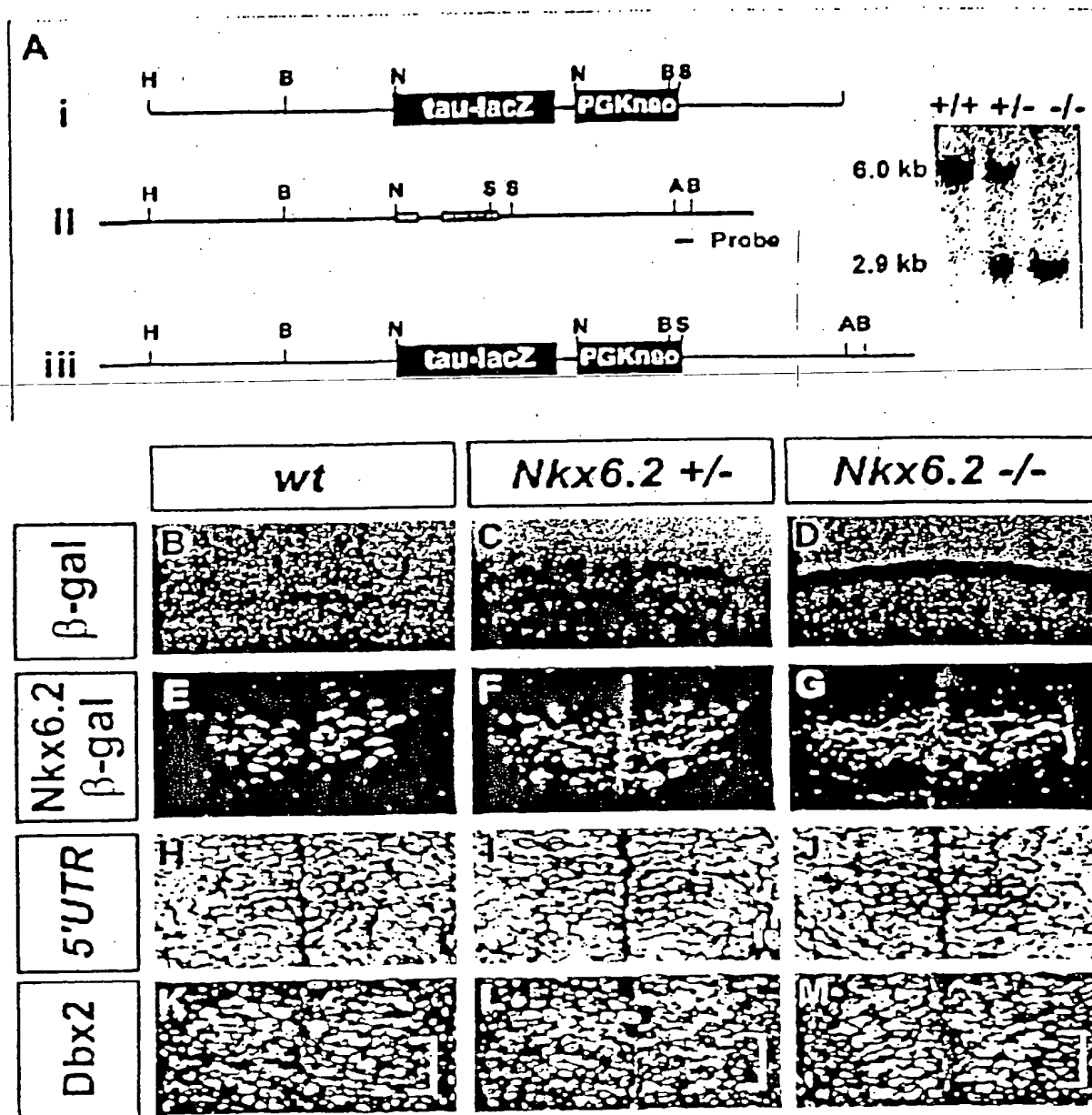
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FIGURE 10



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FIGURE 11



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FIGURE 12

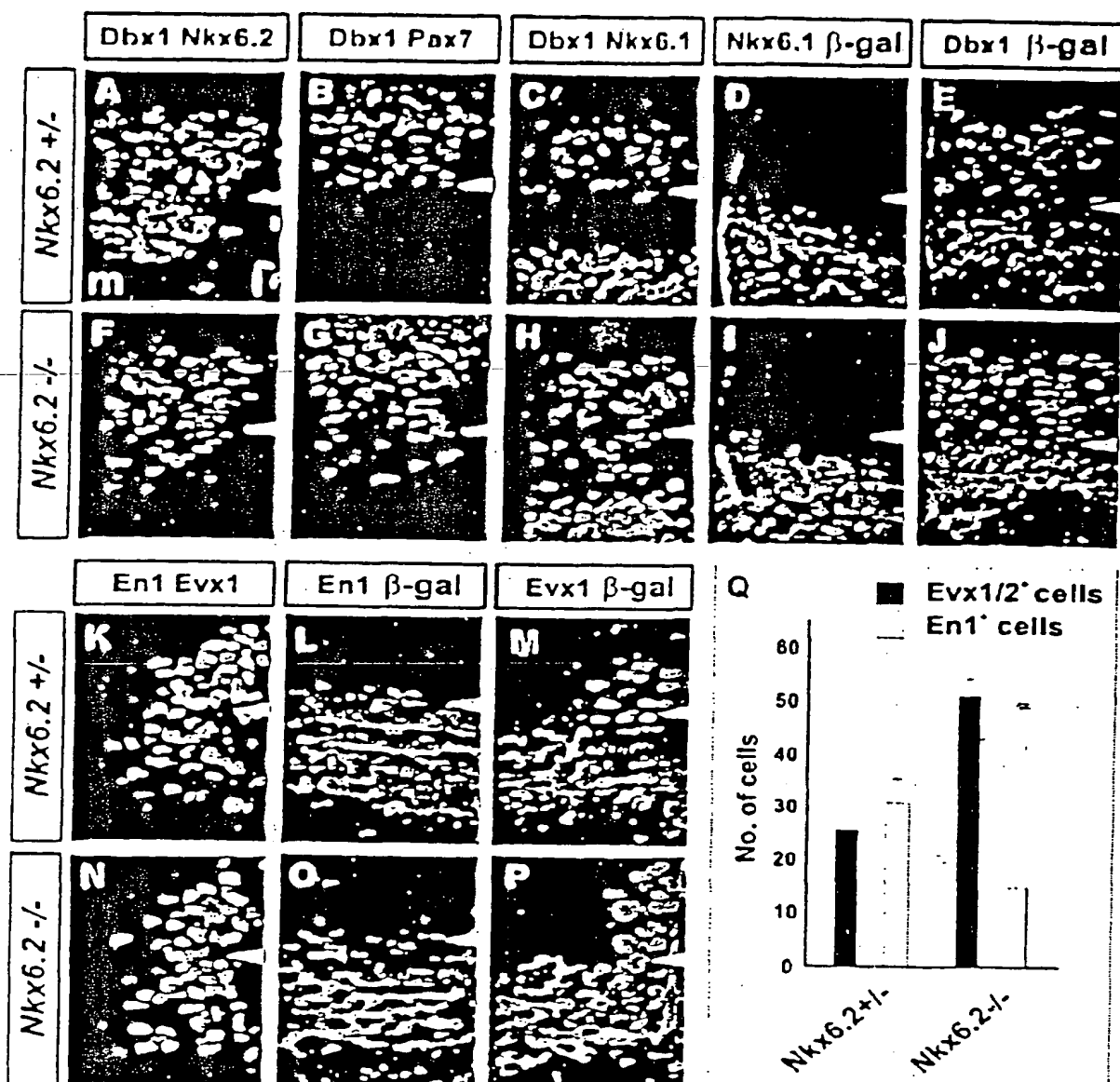
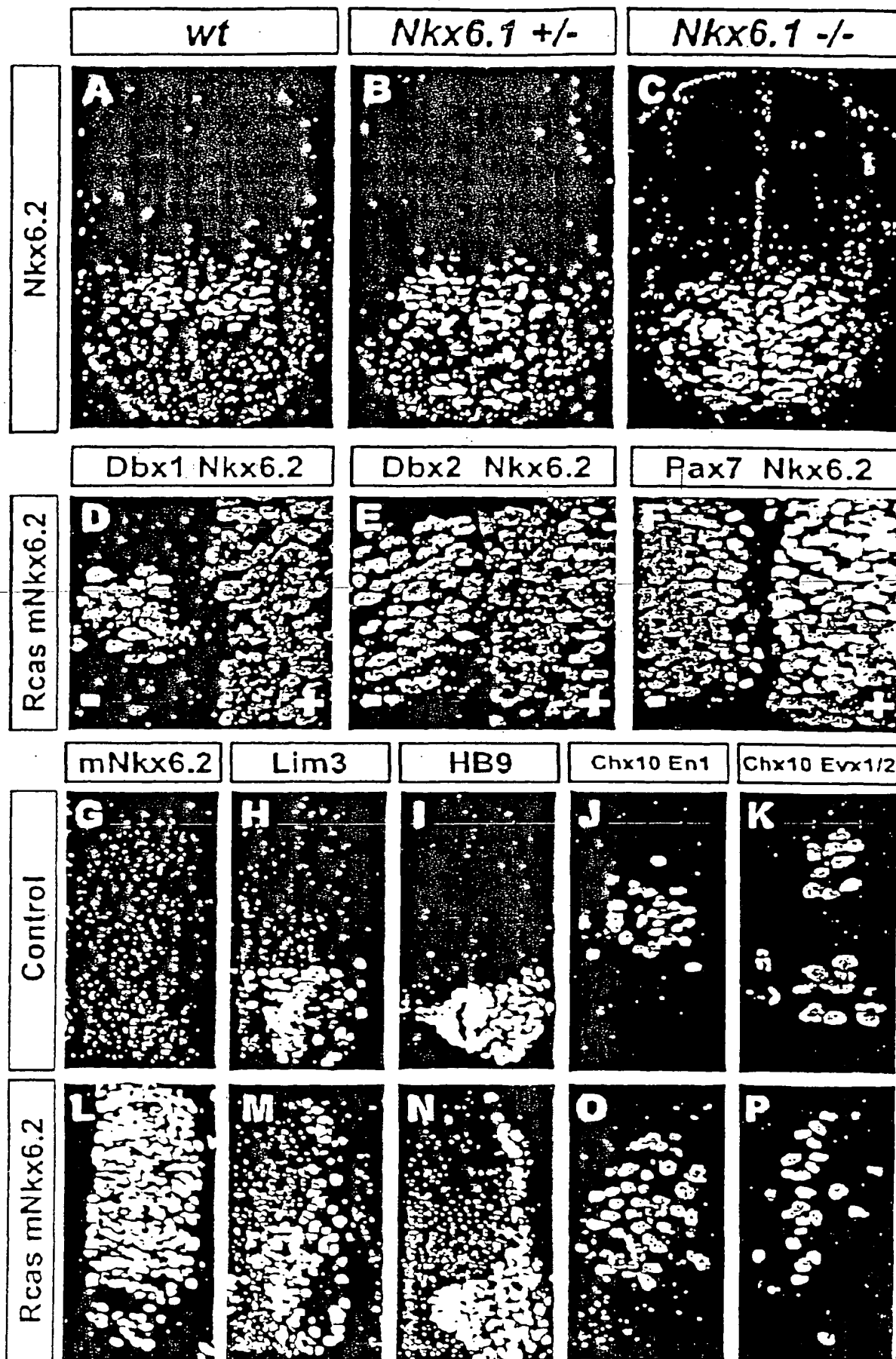


FIGURE 13

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FIGURE 14

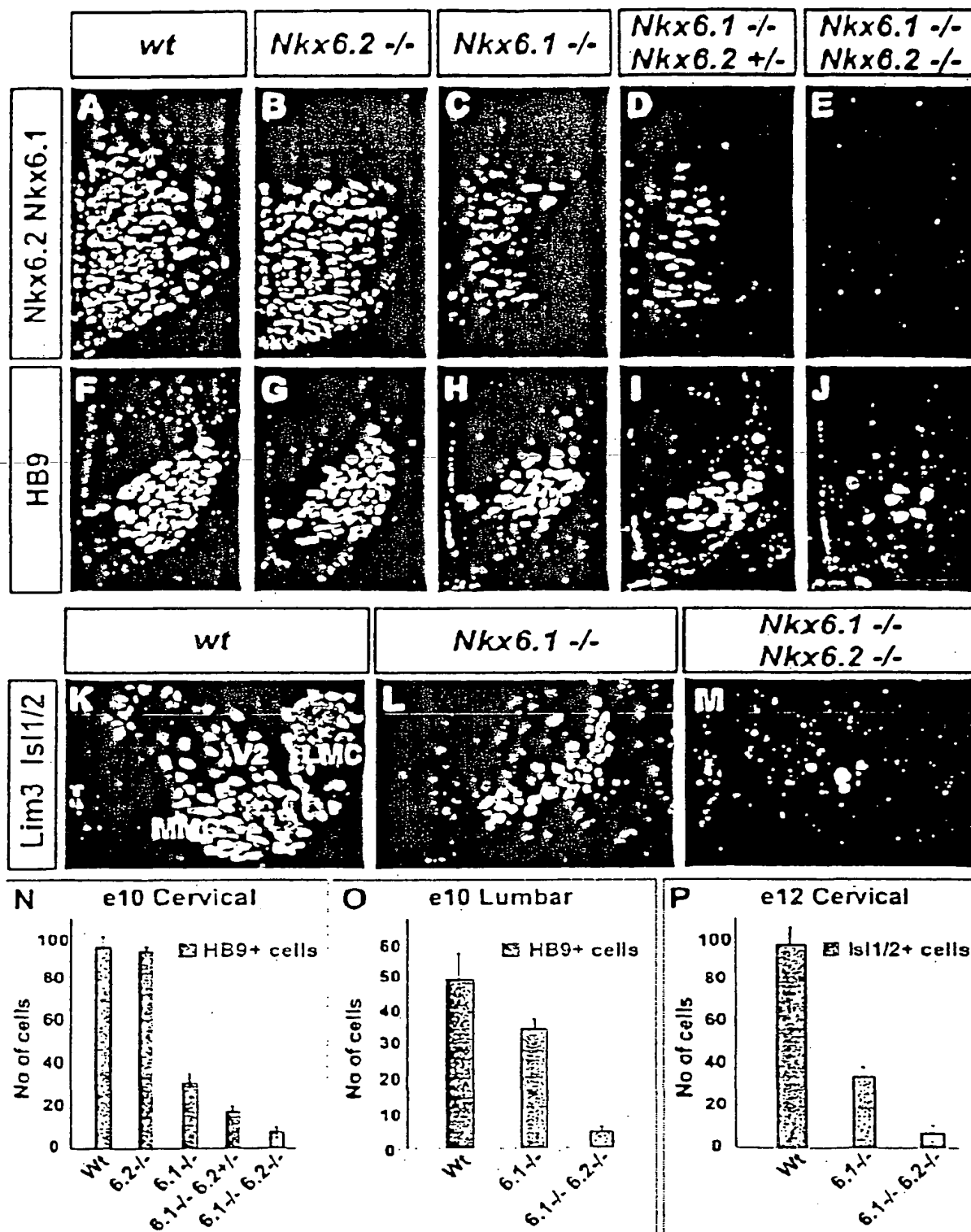
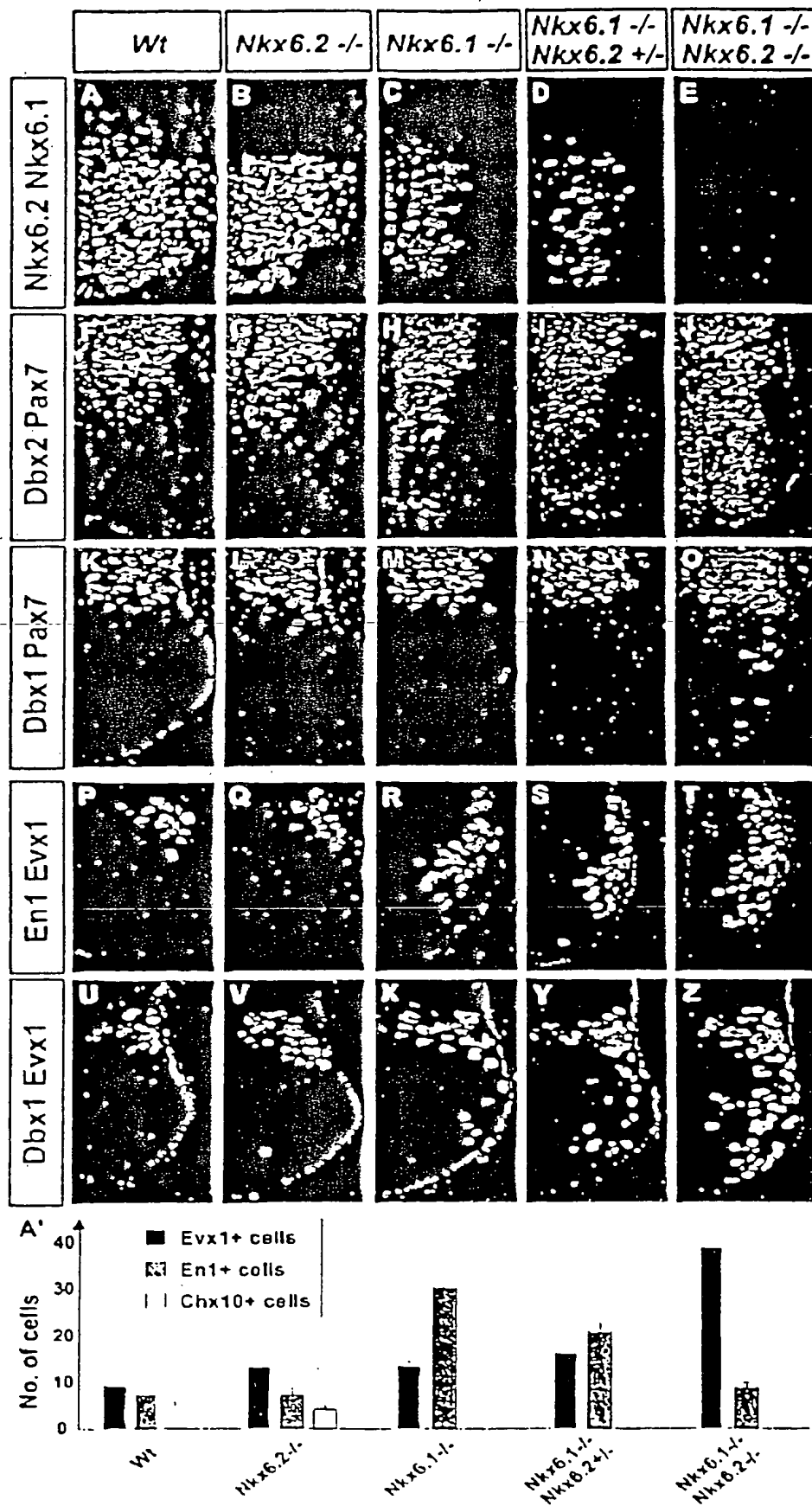


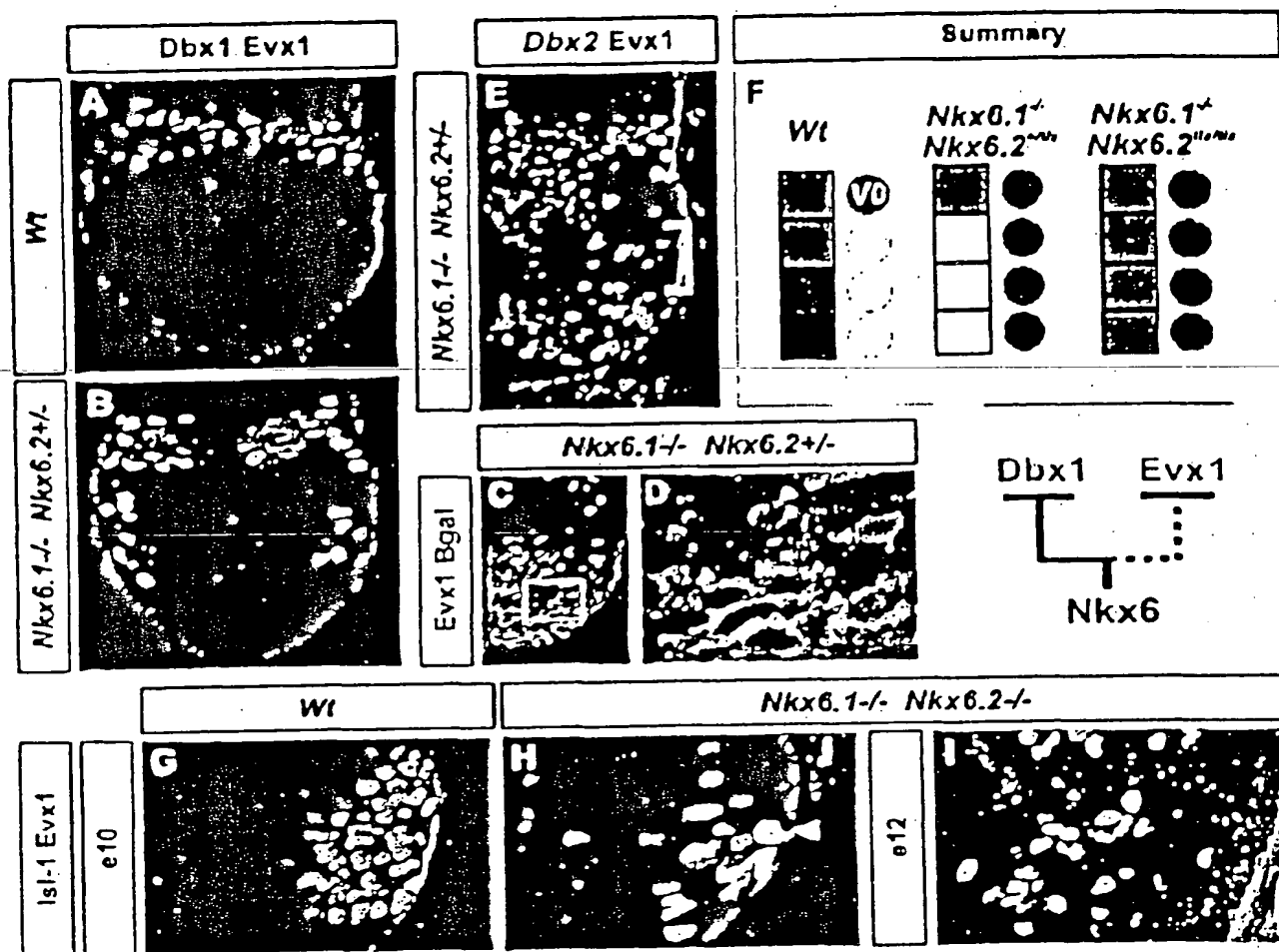
FIGURE 15

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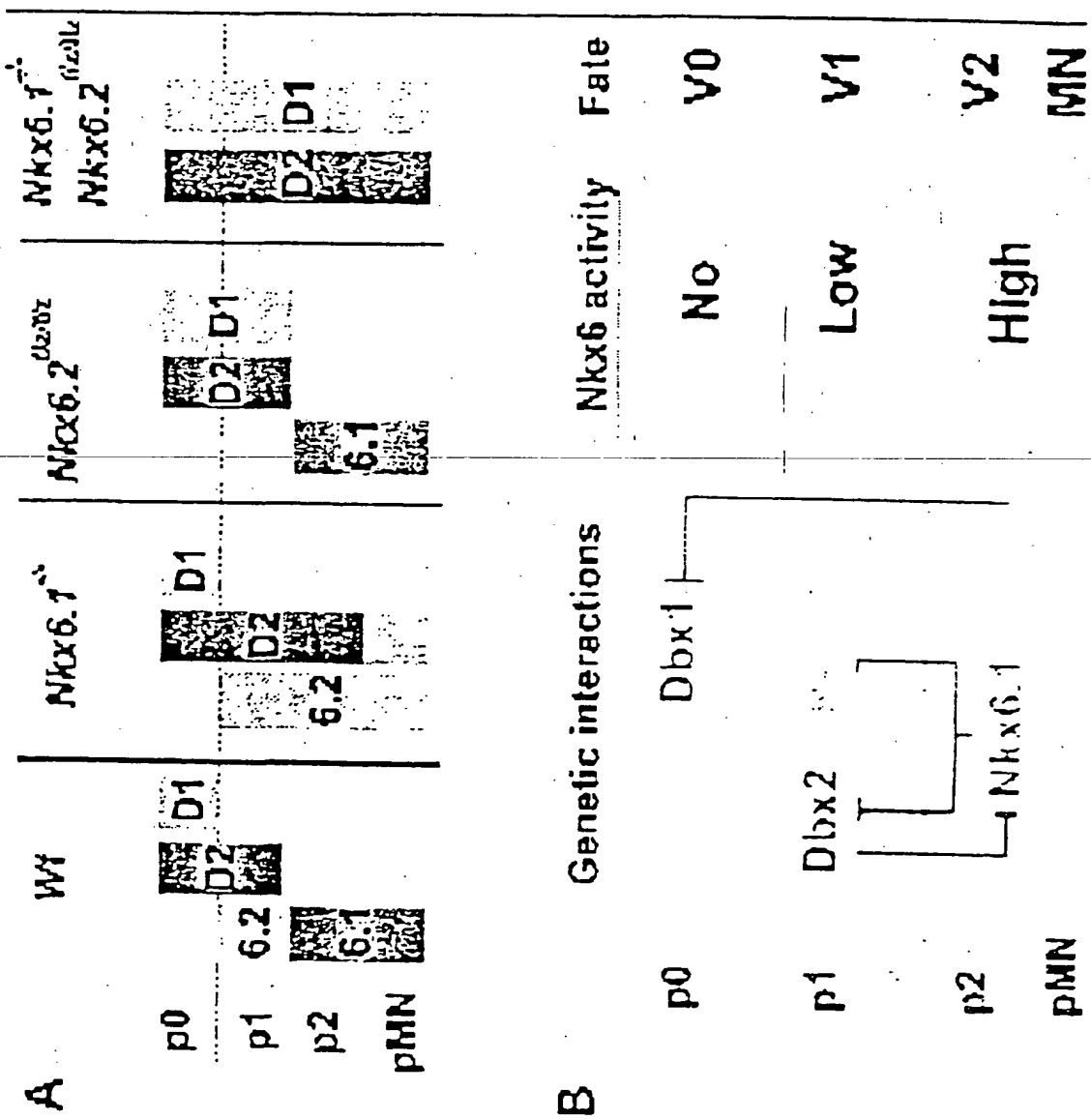
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FIGURE 16



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FIGURE 17



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FIGURE 18

| | | | | | | |
|------|-------------|-------------|-------------|-------------|-------------|-------------|
| 1 | aagcttgccc | tgtctgcacc | cagcaccccc | gggtgtcctc | ctgggagggc | tggaccttgt |
| 61 | ctcttggcag | cacogtgggg | ccctcagagc | cctccatcta | gttccgggca | gggcagggcc |
| 121 | ccttcccaac | accatcggct | gcctctggtc | actcccaccc | aggggcacag | ggaatctctg |
| 181 | aacacccctt | ccctggggag | caggaagact | tgaacacctc | ttggccaggc | cagggcggtt |
| 241 | tctactgtgc | ccaccagacc | cccaggctaa | gccagcaggg | agactggaag | gccagtgctc |
| 301 | cagccccctg | cctgcctcag | ggtggctgct | cgccctctcc | cctcccaccc | cacctggaca |
| 361 | gcctcgggtc | tcagggcgct | gtanggagt | aggcacctgt | gggatggagc | ctcagcgtgg |
| 421 | gtgggagaga | tgtgcaggg | cccagggtgc | gagccgcgtg | tgaacgggga | ggcggcccg |
| 481 | agcgtctccg | tcacgagaag | gaagtggatg | ctcgtgacaa | cagaagaact | cggttcgggg |
| 541 | gaagaacttg | gttggttcagg | ggaagaactc | ggaccattct | ctgtgtgtct | ctctctgtgc |
| 601 | tttcggttaag | tgtgtctccs | ttggctgctg | catttacaag | tgacttttaa | agaacagaag |
| 661 | ctggaaggaa | acctggggct | caggattcag | ggagggggcc | ctgcaagggt | ggagggggccc |
| 721 | ggccaggggc | caggctgtgc | aaggaaactc | aggcccagtg | aagctagagg | gtccacaaag |
| 781 | gctgggcagg | ggccaccctg | aaggggtgct | cagagagttc | aggcaagctc | tccctccctt |
| 841 | gccatccagg | tcctcccagc | ccctgccttc | ttagccccc | ttcagggcct | cctcaaccog |
| 901 | cgggtgttcc | agattccaca | gcctggccca | tatcttccag | ggagagtgtt | cgggtgccc |
| 961 | ggcaccact | gtggccccac | cccagcttca | tcaaagcctc | cctccctgtc | ctgggccaht |
| 1021 | cggcctgggg | aaaagcggca | ccctctcccc | agagcctgat | cctccamtte | atgtggacct |
| 1081 | gtaggtgttg | gcaagtgggc | aagaggccgc | catagcctgg | gaagaggggg | cacctggagc |
| 1141 | ccccacctac | agctgggtac | cccaaaagct | gccgggctct | acctggacac | cctccagctc |
| 1201 | aggagatggg | gtgggggtga | gtttgggtcta | aacagcaaga | cctcaggctc | agctgggaaa |
| 1261 | tgccacggcg | ccaggcccca | catccagcat | gtcctgtggc | tcagggttcc | tggaggcacc |
| 1321 | tccacagtac | ctgtctctcc | cggtgggaag | tcagggtgcg | ggtcctccct | ctccaaccog |
| 1381 | cacccggtct | ctgaaaattg | ctctgaggcc | tgcagctgtc | acacttgcgt | tcattcaccc |
| 1441 | acccagcagc | atgaattcag | tcctggaggc | gccagagga | cagagccctt | gcattccatcc |
| 1501 | atgtcctgag | caaggtggcg | aggaggcgga | catcagacac | atccactaat | gccttcggca |
| 1561 | gggggccaqt | gccaagaggg | gcagccgtgc | tgagtgggag | tgtgggggct | gcaccagacc |
| 1621 | gggtggccag | ggaggcgtcc | ctcaagctga | gaccgcgtgg | aggaggtgag | ccctgtgaag |
| 1681 | tgggggggca | gagtggccgg | ggtgggtgag | cctggggggc | acgaggggac | cagtggaagg |
| 1741 | cctggcaggc | atgggatggc | attaggtgga | aacaggtgga | ggtggagact | cgcatctct |
| 1801 | gaaataaagc | cgctgctagc | aggctgggtc | tcagcaggca | gtgctggaag | tgtgagaagg |
| 1861 | ggccaggcta | ggccaggatg | aggagtggag | cctcctctgc | ccacctaggg | gcgtcaactc |
| 1921 | ccacccctgg | gcggtcccca | ccccagccct | cagcgtcat | ggcctttcag | acccggctgg |
| 1981 | gtccatgagc | ccagtgggac | gcccgggctg | cctggctggg | atctgcgcct | gctccagc |
| 2041 | cctttccccc | tgccctggca | gggtgcctcc | cagagggcac | gggagatggg | gttggggtct |
| 2101 | gtcctgcgtg | ggaggcaggg | ccccttcgag | ttgtgtgtg | gggtggggtt | ttctctagcc |
| 2161 | ccccttcccc | ttccagcaat | tccagagcgt | cctgggtggg | tcctctgttt | ccaagcaaca |
| 2221 | gaaggcaccc | cgcctggggc | cgggtcctct | ggggctcctg | taacccccac | ccgtgccttc |
| 2281 | cgtgggtggg | gccacagag | gggtcccttg | agtcatcttg | ggcctttttt | ggtcctttgg |
| 2341 | tcatgaggac | cccagggagg | cccgtctgt | gtctggaatg | cctgggtgcg | ttacctgttc |
| 2401 | aagcctggag | aggccgggaa | tgcgtcact | tcgggaaaaa | agacaatgca | gggcctttgc |
| 2461 | cggaactgc | taggagaccc | ccggcctggg | ggcgcggtca | gggcggggcag | cttggaact |
| 2521 | cgccataggc | tgcgcgggac | aagtcacctc | agtgataaat | cagagtttgt | gaactctatg |
| 2581 | gcctgggcgg | ccgaaggcga | acgcaggctc | cttccctctg | tggagtcccc | ccgtcgcccc |
| 2641 | tcagccccc | gcgcggggac | accggggcct | argccggctc | tccttcggg | ccgacacccc |
| 2701 | cgcgctctc | cccgtcgccc | gctcccctgc | agacgcgcg | ggggtgccgg | gggagcgcgt |
| 2761 | ttgtctgctt | gacccgcccc | cgcccgggcc | ggagcccgct | gcgttcacgg | tgcaccccc |
| 2821 | ggacagcccg | ggcgcggtaa | gagccccaca | aatacaggct | gaacgagtaa | aacaaacttg |
| 2881 | aatggcctct | gccaaaaccc | gcgctctcgg | ttttccarcg | cggagcgttc | gcgcgcatgc |
| 2941 | cggcagcctt | cctcgcgga | catctgctcg | cgggcccagga | ggtggcatcg | cggaaccttc |
| 3001 | caggcagcgg | ggccgcccgg | cggcgagctc | caggcaagga | catcgcggtc | kgaggcgttc |
| 3061 | ggaccttccc | gggaggagg | ggagtgcct | cggtggtttc | cgagagggcg | gcaccggggg |
| 3121 | acgcaggaga | aaaggtgcgg | gcggggggcg | gagagggggc | ggggccccga | gtgggcggcg |
| 3181 | ggaagcgtag | gaggtgaagc | caccggaccc | acgcgcagct | cggcgaggag | gagggcgga |
| 3241 | aagcgcgtgg | ggcgaggcc | ggggagccgg | gtaggacgcg | gcacctgcgg | agcgcgcaa |
| 3301 | gacttccacg | gcttacaaga | acgtgggaga | gggaccccc | cttaccggc | tcctctgcgc |

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FIGURE 18(CONT.)

| | | | | | | |
|------|-------------|-------------|-------------|-------------|------------|-------------|
| 3361 | ccccaaactca | ccctggcccc | tcatcccgcg | cccctgagcc | ctggagagcc | gcgcgctccg |
| 3421 | cagccagtg | cacgccagcc | ccaccgcgca | ccccacgcgt | tccctcggca | gcccaggag |
| 3481 | gaccgggggg | cgcagacaga | cccagggttt | atcgggccgc | agcgcagcgc | ctccaggtcc |
| 3541 | atgtttctct | agccataaaa | cagcgctagc | gacgccccct | gccccgcccc | tccaggctgt |
| 3601 | gagaagagcc | agagcctgtc | ccgagcgccg | cttctctccc | ccgttccgcc | cgcgcgcgcc |
| 3661 | tectgggct | cagttcggga | gacccgcacc | cctcccggcc | cgcccagggt | ctgcccggcc |
| 3721 | gcgcagagt | gggatccca | gggcgacagc | agcccccgcc | cccaactccc | cctgcccgcc |
| 3781 | ccccccggcc | ccgcagttcc | cgcgtctcag | ctcagagccc | gagccttggg | cgcgggcgcc |
| 3841 | gtcgcccttg | ggtgctggga | ggggcccga | cccgaacccg | gagggccctt | cctcgtgtct |
| 3901 | cctccgagg | agcggggcgc | aggacaggcc | ggggcggttc | tcggggcccg | acgggcgctg |
| 3961 | ggggttccc | ggccaaggct | cgcgggggcc | gatcaccggt | ggggcgccgc | cgcccaatcg |
| 4021 | aatcccaatc | ccagtcgaat | cgagtcggga | gtcgacgggg | gaagcgaaac | ccccgtgaa |
| 4081 | gcggggctgc | acctcagtg | agccggaaag | ccgcccgggc | agccccgagc | gcgcacacac |
| 4141 | ccggcgggcc | caccactgcc | ccggagtttg | gcccagagtg | gcttttccaa | gccgccatcc |
| 4201 | aggagcgccc | gacggcgcca | agtccccgce | tcgacctgca | caaaacgaaa | acggacgctg |
| 4261 | gagggggggc | agggggcgga | cgtgagaccc | cgccccgaa | ccccgggcgc | cgcttctctc |
| 4321 | cgcggcacag | gcccgaagaga | ggccagcgag | cgcgcttccc | tgcgcacaga | ctcgggtccc |
| 4381 | ccacgagcgc | tggggccacag | ccacagccgc | cccgtgtccc | tazatcaata | cgagacgtca |
| 4441 | ccacagacgt | cggagcgttt | gctcgcgccc | gcccgtgcgc | gggctcgag | tcatctcacc |
| 4501 | gcccggctct | cgggatggat | gagcgcagcg | ctcccgggtc | cgtggggcg | ggggggacac |
| 4561 | gtgccccccc | cgcgcgtcta | aggccgctgt | ttgcccgtg | cgccccagc | cgccccacac |
| 4621 | gttcggggccc | tggacagggc | ttccgcgctg | aggccgtcct | ggtctdgtt | ctcccgccgc |
| 4681 | gggattcgcg | agaggcggcc | cgtgggcgaa | gtcgtgggcc | caggtcacat | cctgggggac |
| 4741 | ccccagcggg | agacctggag | gccgatgacg | gggaagtgcc | gagccgcgcg | tgtggtcccc |
| 4801 | ggacccgcct | ccccgccccg | ctcccgcctg | cctcactcct | ccaccgcgcg | ggcgcgctgt |
| 4861 | gcgcgaaacc | agaggcagct | ccgtgcgagc | ctcgccgggc | cgtgagggcc | gtgattccg |
| 4921 | tggactcgag | gcccgcgtcc | tccgcccctc | tgtggccccg | acctgcccgc | agcgcgttcc |
| 4981 | ccgcccgcgt | ccgctgcccgc | tcacacccac | cccagccacg | ggcgggggag | cagtgcgcac |
| 5041 | tgggacgcgg | gcccgggactc | ttccccagat | ggggcgctcc | gagcgcgcgc | gcccgtcctc |
| 5101 | aaatctgcat | tctttccggt | aataaaaatac | gttctcgat | tttttcccta | tttcgcatga |
| 5161 | aaacctttgc | ctaactacac | tcccatccaa | gcgggattta | tttcgtcccc | ggggagataa |
| 5221 | atcggggcga | atttacagcc | cgggagggcac | ctgcccgcgt | aatgggccc | tcatgagtg |
| 5281 | cgcggccggc | ggggggcgcc | ggggcggggg | ggggcgccgc | ccaatggccg | gaccgcgggg |
| 5341 | tccgcagcca | atcagcgccg | gcgcgcgcgc | ccggcgagc | ccccgttatc | agcgcgtccg |
| 5401 | tcccgcgcgc | cgccgcctcc | accggccccc | ggagccgcgc | ccgcgcgcgc | ccgcgcgcgc |
| 5461 | gccccgcgcg | ggagccgcgc | gccccgcgcg | cgccgcgcgc | gccccgcgcg | gcagccgacg |
| 5521 | cccgcggggg | ccgcgcgcaa | acttcccggg | ccggcgggca | ggggcgggcg | cggcgggggc |
| 5581 | cggatgggag | cccggggcgc | cggcgggcgc | gcccattggac | actaacccgc | ggggcggtt |
| 5641 | cgtgctgagc | agtgcggcgc | tggccgcgct | gcacaacatg | gccgagatga | agacgtcgct |
| 5701 | gttcccctac | gcgctgcagg | gtccggccgc | cttcaaggcg | cccgcgctgg | ggggcctggg |
| 5761 | cgcgcagetc | ccgctcggga | ccccgcacgc | catcagcgac | atcctggggc | ggcccggtgg |
| 5821 | cgcggcgggc | gggggacctc | tgggggggct | gccccggctc | aacgggctcg | cgtcgtccgc |
| 5881 | cggggtttac | ttcggggccc | cggcgctgtg | ggcgcgcgcc | taccccaagc | ccctggccga |
| 5941 | gctgcccggg | cgcccgccea | tcttctggcc | cggcggtgtg | cagggcgcg | cctggaggga |
| 6001 | cccgcgtctg | gctggcccgc | gtgagtgccc | cgcgcggggg | gtcggggcg | ggtgggcgcg |
| 6061 | gagggggacc | ccgcgcggcg | ctgaacctcc | tcccttcccc | tcccttgag | ccccggccgc |
| 6121 | cggcgtccg | gacaaagacg | ggaagaagaa | gcactcgccg | ccgaccttct | cgggcagcga |
| 6181 | gatcttcg | ctggagaaaa | ccttcgagca | gaccaagtac | ctggcgggcc | cggagcgcgc |
| 6241 | gcgtctcgcc | tactcgctgg | gcatgaccga | gagccaggtg | aaggtgagcg | cggcggggct |
| 6301 | cgggagagca | gagccggggg | cccgcgtcct | gcgaacggcc | ccagcgccag | ccccggggcc |
| 6361 | cgcggccgcc | tgaccgcccc | gtccactccc | aggtctggtt | ccagaaccgc | cggaccaagt |
| 6421 | ggcgcaagcg | gcacgcggcg | gagatggcgt | ggcccaagaa | gaagcaggac | tcggacgcgc |
| 6481 | agaagctgaa | ggtgggcggc | tcggacgcgc | aggacgacga | cgaatacaac | cggccccctg |
| 6541 | accccaactc | ggacgacgag | aagatcacgc | ggctgctca | gaagcacaaa | ccctcgaaact |
| 6601 | tggcgctggt | cagcccgtgc | ggcgccggcg | cgggggacgc | cttgtgagga | cccgcggggt |
| 6661 | gggggcgaat | ctatttttgc | agaattccgg | ggcgcccccg | ggtgggcgcg | agtgcgtttg |
| 6721 | tatcatcaat | aaattattta | acgggtcccc | gtcgagccgc | tcgctccgga | gcctgcggcg |
| 6781 | cgtgtttctt | ccgtctcgaa | cccggagcga | ggcgccccct | ccccggcccc | ggcttcgccc |
| 6841 | ctgcgcccgc | ctcgggtcct | ccgggttccc | ggtgcggasg | ctgcggggcc | cgggcaggcg |
| 6901 | cgaggaggcg | gcgaaggcgc | agggaaaggg | cccggcccgc | gggaaggaa | cgcagcgaca |
| 6961 | gcccgcagga | gcccgggrcg | gmcccgggga | cggagcagca | ggtacggccc | ggcccgctcc |
| 7021 | gcctcggggc | ggattcggac | gcgcttgggg | gttcccga | gggcgggtga | ccgcggtacc |
| 7081 | cgccctcagt | ccccgcggga | ggtttttctt | cttccgtttt | cccgttttgg | ggccacgtac |
| 7141 | tcgttgccac | cgggcacccc | ttcccgcctc | cccaggggct | tcgctctgat | tatttccaaa |
| 7201 | gtccctctgc | gcatcagcgc | atcccatagg | cccgccttgg | gctcagccgc | tggaaaccggg |
| 7261 | tctgatccgc | tgcaaggagg | cccttcggtc | accatcccgc | cagatcttcc | ccggtggaa |
| 7321 | agcagtttct | tccgaactag | gaccgcaaa | agaaatccga | aataattccg | cgcgggagc |
| 7381 | ggcggggcct | cccgtgggtc | acgcgggggc | agggagccgc | aggccccctg | ggcaaggccc |
| 7441 | gcaagcgcgc | agccgggggg | ctcgggggac | ccgtcttctt | gcccctgaa | gcccgcagct |

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FIGURE 18(CONT.)

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7501 cgcgcggggc tgtgactgcg gctgacaaaa cccctccarc ctcccgcarc ctctgttggc
7561 cggggctgcc matccgctgc awcttaatgg gcgtggctgt tgagttttaa tttttaaaaa
7621 ttaaatgtaa ataagatgat cactgcgggtg gtacgatttc tcttggcatt tgcggaagcg
7681 ttaaagggaa atagaaaggg cttaaaactcg gcgcgttttg ttttaggctc ttagcagcct
7741 tctttacaag gaagcaactc gaagggcgaga agcaacgctt ttctgtgggg agccctctc
7801 agctcagagc agaggggctt cttaaagttt tgaggaaggg aaagcgttga tataatcccg
7861 ttttaaaatg ttgagggata aatcctttat tacagtagaa agtccaaaag gctgtgtttc
7921 tcctctcaat gaacggctta gtgttttggg acagcggtgt atacagtga attccaggat
7981 ttctaattgag cttgatctca aataaaggct atacangagg ccgctccctt gagttagcat
8041 ttcaaagggtg gcaggagaag ggaaagggaag aaaaagcaac acggggacta ttttcaccac
8101 ggtcaatttt attgcttagg aaccagacgg gtcacttcca aaggccctc agaacgacca
8161 acagctgaaa cccgcggggc ggactccgtg ttgaaccggc gacagcggca accacagcag
8221 cgacacggac ctgtgtctcc accaagaaca gattccgcag cggacagcag tcaattgcag
8281 tggtagtatt tatccacac aaacacccag ctaatgcctt cacccggtc ccaggaactc
8341 tgtagtgttc taaagtaaaa atcaataaaa acatacattt gtgtttcatc aacagactct
8401 ctaatcacct tctaattgctg tacttactgc tataggagaa aaatatttgc aacaaggtta
8461 tgacatgggt tgtctgtagc ggagcaatga ggaaatgtac agttttgttt ctctttaata
8521 tttttatata cagcccatgt taaaagcagt ttctattgga agcaaactag gctatttcta
8581 tttctcccat gatattattg ttgtaacgta ggatacttgg caccataaaa cagtaacaaa
8641 agacagacaa acggtttaca aaattcttaa aaggtagacc caggctagct ataaacttca
8701 cattcagttc ttaattattac acagaagaac ggcatgggag taacggcccc ctggtgcaga
8761 cgtgctgtgg ggccgatttt acccacgatg gcgaggccat gtgtgttttt tacgaatttg
8821 tgtgttgatg gacacacagc tgagctccta gactccaatg ccgctgctg atgggactct
8881 cctgtgcgtt catactggaa agtatattta gcataagttt tggtaaagatt tataaattat
8941 ttttaaaaaa tatatatatta tatatatatta tatatatata aaaatggaaa gcagctgcag
9001 tgtgattcaa aaaccatgtg acacggcgca gagtcagtgc cgcggaaagg gcatcggcag
9061 agacagaccc ccttgccatg ctccaggcca cgctgccggc cggcagaggg agtgcccgtc
9121 tcggcttccc cagccccctg acacacctcc acctggcaga gggggtccct ggacacagtg
9181 gggggtctct gtgctgaaga agccccctca ctggcaatca ttaaaaaactg aaaactgtga
9241 agtctacggg acagaccctc tttgctgtct attagagttt tgacaacagg actgtgactt
9301 atttaaaaaa aaaaaaaaac caatatattt acttaatgtc acatagacag acgagacagt
9361 gaggtatgtg gggctgtctc ggaatgggtc ggaggctgaa gcgaagtgtg gggctggccg
9421 tctagcaggt ggcgcttggg cgggttctcg atgcagcttt caagagtgcg tatcggtcc
9481 acggctacag ggaggctcac gaagtgtcct ctctgtggcg tggcatctct tcccaccacg
9541 tcactgcacg acacaacact tgtgcacatg ggcatgaggt ttacctggcc cgggcatgat
9601 tcggaaggcc aggaacacgg gcttgtgggt tcccatgcag acgttgggcc caatgtgggtc
9661 ataggtgaca accttctcct cgctctccga ccgcagcacc agctccttgg cagacgggga
9721 catgaggatg cggtcacacc aggtgggcca ccgggtgttc atgtactgct caccctggcg
9781 ggcgtcctca ctgtacgggt agctgggagg gaacgagatg tccagttcat acagtctgtc
9841 cttaaagaca gacaactcct tgtcaaactc caagagcgcg gtgccgttgt tgtctcgaa
9901 aacctcctgg ttgaagtagt cgaagagttt cttttctaac tggagcataa ccttccggtc
9961 gttgtccgac tcacgaaata tgagcttcac cacttcattg gtgtcggcgg cccggaccgt
10021 ctgcatcggt ggttttgcgt agagcgtctc cacgacagac ttggaatcca gccggaagtt
10081 gaaatcacca aatacaaagt aggaaacctt ctcgaaatcg tgatcaatga ttctgtccag
10141 cacgtagccc agtgcttgt gcccgaattc cgagtacacg gaagggttg tttccaggc
10201 gaccagattg gaagcatcat ggaacagatg gatattcacc aagtcaaagg cacagtctgc
10261 aatcacacc tcgtccggat gaagcctttt cttgaccatt tgcaactcgg gaagtgtct
10321 gcgaaacttc tcttctcca gcatgggctg gctctctaag gtatccgagt agatctctt
10381 gccagcgacc tttctatact tcttagcttt aaagtcaaac tggtagatgt tttttaagga
10441 ctcataagaa aaataaaagc tt
```

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FIGURE 19

1 mdtnrpgafv lssaplaalh nmaemktslf pyalqgpagf kapalgggla qlplgtphgi
61 sdilgrpvga aggllgglp rlnglassag vyfgpaaava rgykpplael pgrppifwpg
121 vvggapwrdp rlagpapagg vldkdgkhh srptfsgqqi falektfeqt kylagperar
181 layslgmtes qkvwfgnrr tkwrkrhaae masakkkqds daeklkvggs daedddeynr
241 pldpnsddek itrllkhhkp snlalvspcg ggagdal

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/27256

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/00, 5/02, 15/63, 15/85, 15/87

US CL : +35/+ 325, 366, 368, +55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : +35/+ 325, 366, 368, +55

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| A | BRISCOE et al. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell. 12 May 2000, Vol. 101, pages 435-445, see entire document. | 1-24 |
| A | MIRMIRA et al. Beta-cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. J. Biol. Chem. 12 May 2000, Vol. 275, No. 19, pages 14743-14751, see entire document. | 1-24 |
| A | OSTER et al. Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. J. Histochem and Cytochem. 1998, Vol. 46, No. 6, pages 717-721, see entire document. | 1-24 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
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| * Special categories of cited documents: | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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| "E" earlier document published on or after the international filing date | "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| "O" document referring to an oral disclosure, use, exhibition or other means | |
| "P" document published prior to the international filing date but later than the priority date claimed | |

| | |
|---|--|
| Date of the actual completion of the international search 16 NOVEMBER 2001 | Date of mailing of the international search report 15 JAN 2002 |
| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | Authorized officer <i>Janece Ford</i> ANNE-MARIE BAKER, PH.D. Telephone No. (703) 308-0198 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/27256

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|-----------|---|----------------------|
| A | SCHWITZGEBEL et al. Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. 2000, Vol. 127, pages 3533-3542, see entire document. | 1-24 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/27256

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

WEST

Dialog (file: medicine)

search terms: Nkx6.1, Nkx6.2, briscoe, ericson, rubenstein, sander, stem(w)cell, neuron?